AN ABSTRACT OF THE THESIS OF

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Title: Micropropagation and Regeneration of Hazelnut (*Corylus* Species)

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A micropropagation system was developed for hazelnut cultivars and rootstocks. Culture establishment was affected by explant source, explant collecting season, and type of explant. The highest frequency of culture initiation and lowest tissue oxidation and contamination were obtained from internodes with single axillary buds from grafted greenhouse-grown plants early in the growing season (March). Nodal explants had a higher percentage of growth than shoot tips. For field collected materials, suckers collected in mid-season were the best option. Tissue contamination and oxidation were the main problems for the in vitro establishment of explants from mature field-grown trees. Attempts to decrease tissue oxidation by using forced outgrowth from field-grown trees and treatment of explants with antioxidants were not successful. There was a correlation between explant oxidation and phenolic content. Shoot multiplication was the best on a modified DKW medium (NCGR-COR medium) supplemented with N^6-benzyladenine (BA) (1.5-3

$mg \cdot l^{-1}$). Plants grown on medium with 3% glucose or fructose produced more and longer shoots than those on medium with sucrose. The general appearance and growth habit of shoots were better on medium with glucose than fructose. Shoot elongation varied with genotype. Changes in medium pH from 4.7 to 5.7 did not significantly affect the multiplication rate. Root formation was induced either in vitro by culturing shoots on NCGR-COR medium with half strength mineral salts and 1 $mg \cdot l^{-1}$ indole-3-butyric acid (IBA) for four weeks or ex vitro by a brief dip in 0.2 or 1 $g \cdot l^{-1}$ IBA and acclimatization. High transplant survival of plants was obtained. Internal bacterial contamination was present in micropropagated shoots. A combination of the antibiotics streptomycin and gentamicin was effective in eliminating contaminants from micropropagated shoots, but some phytotoxicity was noted.

Adventitious shoots were induced at a low frequency from stem segments of 'Nonpareil', 'Willamette', and 'Tonda Gentile Romana' and leaf discs of 'Dundee' on medium containing glutamine, thidiazuron (TDZ), and naphthaleneacetic acid (NAA). Adventitious roots were produced from stem segments and leaf discs cultured on medium with NAA alone or high concentrations of α-NAA or IBA combined with low concentrations of BA.

Micropropagation and Regeneration of Hazelnut (*Corylus* Species)

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LIST OF ABBREVIATIONS:

N^6-benzyladenine (BA);

2,4-dichlorophenoxyacetic acid (2,4-D);

Gibberellin (GA_3);

Indole-3-acetic acid (IAA);

Indole-3-butyric acid (IBA);

N^6-(2-isopentenyl)adenine (2iP);

α-Naphthaleneacetic acid (NAA);

Thidiazuron (TDZ).

MICROPROPAGATION AND REGENERATION OF HAZELNUT (*CORYLUS* SPECIES)

Chapter 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

The hazelnut (filbert) is one of the world's major nut crops. Major production areas are limited to only a few areas near large bodies of water with mild, humid winters, and cool summers. World production, about 470,000 metric tons and increasing, is second only to almond (Mehlenbacher, 1991). Turkey has long been the leading producer and exporter of hazelnuts, accounting for 64.6% of the total production with Italy second (22.7%), Spain third (5.3%), and the United States fourth (2.7%) (Mehlenbacher, 1991). In the United States, production is centered in the Willamette Valley of Oregon, where the climate is influenced by the Pacific Ocean (Mehlenbacher, 1991).

All important world cultivars were selected long ago directly from the wide-ranging wild species, *Corylus avellana* L., in Europe and Turkey (Mehlenbacher, 1991). The genus *Corylus* includes several species which are widely distributed throughout temperate regions of the northern hemisphere. The nine most widely recognized include the five shrubby species, *C. avellana* L., *C. americana* Marsh., *C.*

cornuta Marsh., *C. heterophylla* Fisch., and *C. sieboldiana* Blume.; and the four tree species, *C. colurna* L., *C. jacquemontii* Decne., *C. chinensis* Franch., and *C. ferox* Wall. All species have edible nuts which are gathered from wild populations by humans, depending upon the local abundance, the quality of the kernels, the shell thickness, and the ease of extracting nuts from the husks. Variability among *Corylus* species is used to select for improved rootstocks, plants resistant to eastern filbert blight, and those adapted to colder regions.

All *Corylus* species that have been investigated for chromosome number are diploid with 2n = 2x = 22, except for a few aberrant forms. Instances of unusual chromosome number include: 2n = 18 in somatic tissues of *C. colurna* and *C. maxima f. atropurpurea* (Dochnahl) Winkler (Kasapligil, 1968), tetraploids induced by colchicine treatment and a spontaneous triploid among a group of *C. avellana* seedlings (Danielsson, 1946; Danielsson-Santesson, 1951). Botta et al. (1986) discovered three spontaneous tetraploids among a group of *C. heterophylla* seedlings.

Hazelnut is monoecious, wind-pollinated, and self-incompatible. The conventional hazelnut propagation method is layerage, a slow and inefficient method which limits the rapid increase and introduction of new cultivars (Lagerstedt, 1970). While cutting and grafting could be efficient propagation methods, they are not used on a commercial scale because of the bud abortion when rooting cuttings and the lack of suitable rootstocks for grafting. Micropropagation may be an attractive alternative propagation method for hazelnut.

The goals of the thesis were to develop an efficient system to micropropagate hazelnut cultivars and investigate plant regeneration from mature hazelnut tissues.

Literature Review

Hazelnut micropropagation

Micropropagation offers several advantages over conventional propagation methods including: 1). A large number of plants can be produced in a small area, in a short time and in any season. Once the stock culture has been established, shoot multiplication and plantlets can be produced at any time of the year in a small growth room. 2). By conventional techniques, multiplication of a new cultivar of hazelnut takes several years. Micropropagation increases the number of available propagules at a much faster rate so new cultivars can be released rapidly and be available to growers in less time. 3). Micropropagation can be used to produce and maintain pathogen free plant material. 4). It facilitates germplasm storage and exchange. 5). It can be used for studying physiology, pathology, and genetics.

Micropropagation can be achieved through three methods: axillary proliferation, adventitious bud formation, and somatic embryogenesis, by using seedling, juvenile, or adult material. Micropropagation is much more difficult with woody plants than with herbaceous plants (Thorpe and Harry, 1990). Most successful micropropagation of tree species is restricted to explants from seeds or seedlings so information dealing with mature (adult) materials is very limited.

The feasibility of in-vitro techniques to propagate hazelnut was demonstrated by Radojevic et al. (1975) with embryos. Satisfactory micropropagation has been achieved for primary explants of seedlings and juvenile materials of hazelnut but

there are still difficulties in culture establishment and multiplication with adult material.

1. Axillary shoot proliferation

Several major factors affect micropropagation of hazelnut. The source of explant material and technical problems associated with tissue contamination and oxidation in culture establishment are two major concerns. It is also necessary to optimize the composition of the culture medium to establish and maintain axillary bud proliferation. Types and concentrations of plant growth regulators must be selected to induce axillary bud growth and shoot elongation for optimum plantlet production. Finally conditions for in-vitro rooting and acclimatization must be established so that plants can be returned to the field for production.

A). Culture establishment

The source of explants has a pronounced effect on the success of culture establishment. Explants from seedlings and juvenile materials are easier to establish than those from adult materials (Thorpe and Harry, 1990). Contamination and oxidation are problems of hazelnut in-vitro culture which vary greatly with the explant source (Rodriguez et al., 1989b). Shoots from seedling, juvenile, and adult materials have been used as explant sources for hazelnut.

a). Seedlings: Seedling explants can easily develop into shoots (Anderson,

1984; Al Kai et al., 1984; Perez et al., 1985). The establishment of cultures from seedlings presents no serious contamination problem. Sterilization of seedling explants can be carried out in 85% ethanol for five minutes, and sodium hypochlorite with a few drops of Tween-20 for 20 minutes then rinsing with sterile water several times (Rodriguez et al., 1989b).

b). Juvenile and adult materials: Microbial contamination and the acute effects produced by the sterilization agents used are serious problems with juvenile and adult materials (Rodriguez et al., 1989b). It is difficult to establish a satisfactory sterilization method for juvenile and adult materials, even when the selected trees are treated to reduce microbial contamination.

Efforts to reduce tissue contamination and oxidation of adult plant material were made by Messeguer and Mele (1983 and 1987), Perez et al. (1987), and Diaz-Sala et al. (1990). Messeguer and Mele (1983) obtained contamination rates less than 50% from yearly shoots of trees of cv. Negret grown under controlled conditions by disinfecting 2 cm shoot sections with a solution of quinoline sulphate (100 $mg \cdot l^{-1}$) for four hours and rinsing in sterile water, then disinfecting with commercial bleach (16 $g \cdot l^{-1}$) for 20 minutes and rinsing three times in sterile water. They also reported that explants taken during autumn with a large explant size and diameter were important for getting better results. A similar effect of shoot diameter (> = 4 mm) was reported on adult plant material by Diaz-Sala et al. (1990). Perez et al. (1987) achieved 70% initiation of sterile cultures using lateral buds taken from the vegetative shoots of 12-month old greenhouse-grown stock hazelnut plants obtained from cuttings of cv. Negret. Perez et al. (1987) reported a procedure of sterilizing

buds from adult trees. Buds were surface sterilized with 95% ethanol for five minutes and immersed in 1.5% sodium hypochlorite solution for 30 minutes and then rinsed three times with sterile water. Similar methods were used for explants from mature trees to eliminate contamination (Diaz-Sala et al., 1990; Bassil et al., 1992) and were successful, except for buds collected in the fall after the start of the rainy season (Bassil et al., 1992). Bassil et al. (1992) reported that surface-sterilization with ethanol increased the formation of phenolics and browning of buds. Diaz-Sala et al. (1990) found that newly developed shoots from the forced outgrowth of cold-stored field-grown branches greatly increased the morphogenic capacity of explants and reduced contamination and oxidation of tissues.

B). Shoot multiplication

a). Basal culture medium: Many modifications of the medium, mineral nutrient composition and various vitamins, have been tested. Most researchers had good multiplication with seedling and young materials but a low multiplication rate with adult materials.

Anderson's success with explants from seedlings (3-5 shoots/explant) was achieved by revising the MS inorganic salt formula (Murashige and Skoog, 1962): reducing KNO_3 from 1900 to 480 $mg \cdot l^{-1}$ and NH_4NO_3 from 1650 to 400 $mg \cdot l^{-1}$ (maintaining the same ratio of nitrate to ammonium nitrogen) and replacing KH_2PO_4 with NaH_2PO_4; the iron and iodine were also modified (Anderson, 1984).

Al Kai et al. (1984) achieved their best results for shoot proliferation with

seedling materials by adding Zucherelli's vitamin solution to MS medium. Substitution of Fe-EDTA with Fe Sequestrene (Ciba-Geigy) was found to enhance the green color of shoots (Al Kai et al., 1984), a result confirmed by Bassil et al. (1992).

Perez et al. (1987) used half strength K(h) medium [Cheng's mineral salts (Cheng, 1975) with per liter of 0.25 mg thiamine, 0.25 g inositol, 30 g sucrose and 7 g bacto-agar] for shoot multiplication using embryos, shoot and cotyledonary node segments of hazelnut seedlings and single lateral bud explants from adult trees of cv. Negret. They had good results for multiplication with materials from embryos and seedlings, but only one shoot was produced from each lateral bud of adult explants.

Diaz-Sala et al. (1990) grew nodal segments and apical buds of adult hazelnut cv. Tonda Gentile delle Langhe successfully in modified MS medium (half-strength nitrates but double strength calcium chloride and magnesium sulfate, plus 2 $mg \cdot l^{-1}$ ascorbic acid). They also found a double-phase culture system to be better than a single solid-phase system for shoot proliferation and elongation. Another modified MS medium with a high Ca level was very effective for shoot multiplication with buds from mature trees (Bassil et al., 1992).

b). Plant growth regulators: Among the plant growth regulators, N^6-benzyladenine (BA), alone or with other growth regulators, is the most effective shoot proliferation inducer for hazelnut explants from all plant sources (Anderson, 1984; Al Kai et al., 1984; Perez et al., 1987; Diaz-Sala et al., 1990; Bassil et al., 1992). The optimum BA concentrations used by these authors varied from 0.1 to 5 $mg \cdot l^{-1}$. Perez et al. (1987) found that BA concentrations of 0.1-2 $mg \cdot l^{-1}$ was suitable, while

8-10 mg•l^{-1} inhibited shoot elongation of adult explants and 4-6 mg•l^{-1} BA resulted in abnormal development. Diaz-Sala et al. (1990) compared the effects of several concentrations of BA on the growth of buds from adult hazelnut cv. Tonda Gentile delle Langhe and found a lower percentage of bud response at low concentrations of BA (0.5 mg•l^{-1}) and a higher percentage of bud outgrowth at high levels (1-5 mg•l^{-1}). For shoot proliferation they employed 5 mg•l^{-1} BA combined with 0.01 mg•l^{-1} indoleacetic acid (IAA) and 0.1 mg•l^{-1} gibberellin (GA_3) in a modified MS medium. Bassil et al. (1992) observed more shoot multiplication with 5 μM (1.1 mg•l^{-1}) BA than 25 μM (5.6 mg•l^{-1}) for cvs. Ruby and Ennis and selections USOR 8-73 and USOR 1-70.

Other cytokinins are rarely used and kinetin and N^6-(2-isopentenyl) adenine (2iP) are less effective than BA (Messeguer and Mele, 1987; Perez et al., 1987). For seedlings, Anderson (1984) found that 2 mg•l^{-1} BA combined with 1 mg•l^{-1} 2iP was best for budbreak and vigorous shoot growth.

The role of auxin and GA_3 on shoot proliferation of hazelnut is not well established. In some cases, low levels of IAA or naphthaleneacetic acid (NAA) (0.01 mg•l^{-1}) and GA_3 (0.1 mg•l^{-1}) were supplied to the medium (Al Kai et al., 1984; Diaz-Sala et al., 1990). GA_3 (0.1 mg•l$^{-1)}$ facilitated shoot elongation in Tonda Gentile delle Langhe (Diaz-Sala et al., 1990). In other cases, neither auxin nor gibberellin were used (Bassil et al., 1992; Perez et al., 1987).

C). Rooting and acclimatization

Roots can be induced when in-vitro shoots are cultured on medium containing IBA or dipped in an IBA solution and then grown on auxin-free medium (Anderson, 1984; Al Kai et al., 1984; Messeguer and Mele et al., 1985; Perez et al., 1985, 1987; Rodriguez, 1989b; Diaz-Sala et al., 1990; Bassil et al., 1992). The optimum concentration of IBA in the medium varies. Anderson (1984) obtained 65% rooting of plantlets from seedlings rooted with 0.5 $mg \cdot l^{-1}$ IBA on Anderson medium. Al Kai et al. (1984) emphasized that high concentrations of IBA induced callus formation (1 $mg \cdot l^{-1}$) or plump roots (0.5 $mg \cdot l^{-1}$) while low concentrations stimulated root formation and shoot elongation (0.1 $mg \cdot l^{-1}$). Rodriguez et al. (1989b) noted that increased IBA concentrations promoted root formation, but also necrosis. An inverse relationship between root number and root length was found by Bassil et al. (1992). These differences in root induction might be attributed to the differences in cultivar chosen, period of time in contact with the root inducer, length of shoots, and the basal medium used. IAA was also effective in stimulating rooting with seedling materials (Anderson, 1984).

Dipping shoots in an IBA solution and then transferring to auxin-free medium was found to be a more effective method of root induction than growing shoots on medium with IBA and resulted in 100% rooting of micropropagated shoots (Bassil et al., 1992). Rooting of 80% of shoots was achieved by immersion of 5-mm of the basal end of shoots for 5 days in half-strength K(h) liquid medium containing 10 $mg \cdot l^{-1}$ IBA and their subsequent transfer to solid half-strength K(h) without IBA

(Perez et al., 1985). Perez et al. (1987) observed 75-80% rooting from juvenile material and nearly 50% from adult material by immersing 2 cm long shoots in 1-5 $g \cdot l^{-1}$ IBA solution for 10 seconds and then transferring to a basal medium. Rodriguez et al. (1989b) achieved more than 80% rooting by the immersion of the shoot base in 1 $mg \cdot l^{-1}$ IBA solution for 2 min., but root origin was unpredictable and sometimes plantlet survival decreased. Diaz-Sala et al. (1990) obtained 100% rooting by immersion of the shoot base in IBA solutions (0.1-1 $g \cdot l^{-1}$) for 10 seconds followed by a 20-day culture on medium without growth regulators. There are no reports of ex-vitro rooting of hazelnut.

For plant acclimatization, high rates (93%) of plantlet survival were achieved by planting rooted shoots in a 1:1 mixture of Redi-earth and perlite (Anderson, 1984).

2. Adventitious shoot proliferation

Adventitious shoots are those shoots induced from a tissue which normally does not produce them. They can be produced directly on the explant or on a callus derived from the primary explant. Callus formation of hazelnut has been reported but without adventitious shoot regeneration (Radojevic et al., 1975; Perez et al., 1983b).

The establishment of a continuous callus culture is readily attained in hazelnut. Radojevic et al. (1975) described a callus culture of hazelnut from isolated immature embryos grown on a modified MS medium, composed of the mineral

solutions of MS with (in $mg \cdot l^{-1}$) 200 casein hydrolysate, 100 myo-inositol, 2 thiamine, 5 nicotinic acid, 2 adenine and 10 pantothenic acid plus 1 $mg \cdot l^{-1}$ kinetin and 1 $mg \cdot l^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D). The callus tissue was capable of somatic embryogenesis throughout two years of culture. Cotyledons from seedlings cultured on MS or K(h) medium in the presence of various concentrations of IBA, GA_3 and 2,4-D alone or in combination with kinetin produced callus with different characteristics (Perez et al., 1983b). MS medium was suitable for callus induction and 1 $mg \cdot l^{-1}$ 2,4-D induced hard and nodular callus. Higher 2,4-D concentrations caused phenolic production and after some time the callus tissue turned brown and died. Callus formed in the presence of NAA or IBA was more fragile than that in 2,4-D, grew rapidly without browning and was usually morphogenic. The addition of cytokinins (kinetin or BA) was important for callus establishment and proliferation (Perez et al., 1983b). Callus formation also occurred when embryonic, juvenile, and mature explants were cultured for other purposes (Perez et al., 1985; Diaz-Sala et al., 1990; Bassil et al., 1992). There are no reports of adventitious shoot formation other than the observation of Bassil et al. (1992), who speculated that adventitious shoots might arise from the callus produced at the base of shoot explants.

3. Somatic embryogenesis

Somatic embryogenesis in hazelnut has been achieved (Radojevic et al., 1975; Perez et al., 1983a, b, and 1986) and depends on the potentiality of the tissue used as explants rather than on exogenously applied growth regulators (Rodriguez et al.,

1989b). The first report on in-vitro culture of hazelnut made by Radojevic et al. (1975) dealt with embryoid induction. They obtained callus by proliferation of isolated hazelnut embryos on modified MS minerals with 1 $mg \cdot l^{-1}$ kinetin and 1 $mg \cdot l^{-1}$ 2,4-D. The callus tissue consisted of small round nodules which in cross-section could be distinguished as embryogenic structures; early stages of embryoid development were frequently found and the embryoids were morphogenic. The induction of embryoids was not inhibited by 2,4-D, although it prevented further development into plantlets.

Perez et al. (1983a) published the first report on embryoid induction from vegetative tissues. Cotyledonary node segments from seedlings were cultured for 20 days, first in the presence of BA (0.11 $mg \cdot l^{-1}$) and IBA (1 $mg \cdot l^{-1}$) and then with BA (1.1 $mg \cdot l^{-1}$) and IBA (0.1 $mg \cdot l^{-1}$) to induce embryoids. Subsequent proliferation was successfully maintained during five subcultures on a K(h) basal medium and for an unlimited number of subcultures in the presence of BA (0.11 $mg \cdot l^{-1}$). Plantlet regeneration was achieved from 60% of the explants used.

During the first sub-culture, Perez et al. (1986) also observed structures with very heterogeneous morphology (globular, heart-, torpedo-shaped, and cotyledonary embryoids) and abnormal structures coexisting in the same area as the embryogenic clusters. They found that 2,4-D induced embryoids which developed only to the globular stage and inhibited further embryoid development; BA was very important in the recovery of polarized development (Perez et al., 1986).

There are no reports of somatic embryogenesis from mature material.

4. Conclusions and prospects

In vitro propagation of hazelnut has been successfully achieved with materials from seedlings and juvenile plants. Several modified MS media with BA have been satisfactorily used to proliferate plantlets. Initiation and multiplication rates achieved with adult material are not high enough for consideration on a commercial scale. Especially high percentages of tissue contamination and oxidation with adult materials interfere with culture establishment. Further studies on the optimum procedures are needed to improve the in-vitro response of adult material.

Most reports of tissue culture with hazelnut are limited to micropropagation by axillary shoot proliferation. However, more plants might be produced by induction of adventitious shoots from callus or somatic tissues and somatic embryogenesis, provided that somaclonal variation does not occur. The ability to regenerate whole plants from somatic tissues such as leaf discs or stem segments is a prerequisite for *Agrobacterium* mediated transformation of higher plants. The development of methods for plant regeneration from somatic tissues of hazelnut would facilitate genetic transformation of this plant.

Special problems in micropropagation of woody plants

Successful micropropagation has been achieved for various woody plant species. However, most of these deal with seedlings or juvenile materials and some

difficulties are present in the micropropagation of mature materials. Juvenility-maturation, contamination and browning of tissue, hyperhydricity (vitrification), difficulty of rooting, and high mortality upon transfer to soil are the major problems often encountered in micropropagation of woody plants.

1. Juvenility-maturation

The change from juvenility to maturation in woody plants plays a role in culture establishment, shoot multiplication, and root induction (Bajaj, 1991). Most successful micropropagation of woody plants is with explants from embryos and seedlings (Thorpe and Harry, 1990). Culture establishment and/or subsequent shoot multiplication and root induction are difficult for explants from mature trees. Explants for woody plant in-vitro culture are normally chosen from the most juvenile tissues within a tree or parts of the donor tree are rejuvenated by special treatments prior to excision. Various pretreatments have been used for rejuvenation, including severe pruning, spraying with cytokinins or gibberellin (Magalewski and Hackett, 1979; Bouriquet et al., 1984), serial rooting or grafting on juvenile rootstocks (Franclet et al., 1987), grafting on juvenile rootstocks and subsequent spraying with BA, and partial etiolation of starting material (Ballester et al., 1990).

2. Microbial contamination and browning of the explants

Contamination of cultures is a major obstacle in culture establishment from

mature woody plant material. Isolation of meristems or small shoot tips (Hakkaart and Versluijs, 1983) and explants from stock plants under controlled conditions (Messeguer and Mele, 1983) have been used to obtain aseptic cultures. Microbial contamination can be exogenous or endogenous, is not always apparent at the culture establishment stage (Rodriguez et al., 1989a), and may be difficult to eliminate (Pier-Luigi, 1990). The use of chemicals or antibiotics in the medium has not proved to be a reliable remedy against bacteria (Pier-Luigi, 1990). The type, level, and treatment duration of antibiotics used for plant material are rarely studied (Thorpe and Harry, 1990).

Browning of explants at the culture establishment stage has been a serious problem for walnut (Rodriguez, et al., 1989a), chestnut (Mullins, 1987), and other plants (Preece and Compton, 1991). Browning often inhibits growth and development of the explants, may be lethal, and has been attributed to oxidized polyphenols and tannins in the tissues (Preece and Compton, 1991). A wide variety of techniques are used to overcome the problem and obtain healthy cultures (Preece and Compton, 1991; Thorpe and Harry, 1990). These include stock plant treatments (Kerns and Meyer, 1988), proper selection of explants, special care during explant preparation, use of antioxidants (ascorbic acid, citric acid, cysteine, PVP, DIECA) as a presoak or in the medium, use of activated charcoal in the medium, and explant presoaks in sterile water (Chevre et al., 1983; Monaco et al., 1977; Welander, 1988a). In some cases, the choice of another medium (Chevre et al., 1983; Uno and Preece, 1987), changing the level of sucrose in the medium (de Fossard et al., 1978), use of liquid rather than agar solidified medium (Stevenson and Harris, 1980) and changing

or pulsing plant growth regulators (Cooper and Cohen, 1985; Lakshmi Sita and Shobha Rani, 1985), or using basal medium without plant growth regulators for about a week have reduced the problem. Changes in culture environment may be used such as incubation of cultures in low light or darkness (Marks and Simpson, 1990), avoiding high temperatures inside the transfer chamber (Monaco et al., 1977), discarding explants that show browning (Singha, 1980), and either transferring explants to new locations within the same vessel or to a fresh medium within relatively short intervals of time when discoloration is first noticed (Read et al., 1985; Yang et al., 1986) .

3. Hyperhydricity (vitrification)

Hyperhydricity is a serious problem in micropropagation (Gaspar, 1991). Hyperhydric plants appear turgid, watery at the surface and hypolignified, translucent, dark green, and break easily (Debergh et al., 1992). The leaves are thick with large intracellular spaces and poor vascular connections between roots and stems (Debergh et al., 1992; Gaspar, 1991). Hyperhydricity mostly occurs during intensive multiplication due to high humidity and high salt levels, especially ammonium nitrate as in MS medium (Daguin and Letouze, 1986), liquid medium (Druart et al., 1984), and cytokinins (Leshem et al., 1988). Increasing agar concentration and decreasing the levels of cytokinins, ammonium nitrate, dilute minerals in the medium, and loosening culture vessel plugs or cap have been recommended to avoid hyperhydricity (Gaspar et al., 1987).

4. Induction of rooting and acclimatization

Root induction may present a problem for some woody plants. To increase root development in vitro, shoots are transferred to auxin-free medium after a period in a root initiation medium (Dolcet-Sanjuan, 1991). There may be a high mortality rate for transplanted shoots due to the failure of root induction prior to acclimatization (Preece and Sutter, 1991). However, if losses of unrooted microcuttings are low, then rooting and acclimatization can be done simultaneously (Preece and Sutter, 1991). Heavy losses of in vitro plants during transfer from culture vessels to soil has stimulated considerable research on acclimatization. To increase the survival rate of plants, it is necessary to harden off plants before transfer to the greenhouse, use humidity tents (or mist beds), slow change from in vitro to greenhouse conditions, or use antitranspirants (Preece and Sutter, 1991).

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Chapter 2

IMPROVED SHOOT MULTIPLICATION OF MATURE HAZELNUT (*CORYLUS AVELLANA* L.) IN VITRO USING GLUCOSE AS A CARBON SOURCE

Abstract

Shoot cultures established from mature trees of hazelnut (*Corylus avellana* L.) cvs. Nonpareil and Tonda Gentile Romana were used to determine the effects of basal media, carbon sources and concentrations, pH, and cytokinins on shoot multiplication. All factors except pH affected the multiplication rate. Shoot multiplication was the best on a modified Driver and Kuniyuki medium for Paradox walnut (DKW) supplemented with 6-benzyladenine (BA) (1.5 - 3 mg/l). Plants grown on 3% glucose or fructose medium produced more and longer shoots than those on sucrose. The general appearance and growth habit of shoots were better on medium with glucose than fructose. 'Nonpareil' shoots elongated better than those of 'Tonda Gentile Romana.' Changes in medium pH from 4.7 to 5.7 did not significantly affect the multiplication rate. More than 10 genotypes propagated well on modified DKW medium with glucose. This is the first report of the effect of carbon sources on shoot multiplication of hazelnut and provides a basis for further research in the improvement of hazelnut micropropagation.

Abbreviations: MWPMC, modified woody plant medium for chestnut (Yang et al.,

1986); DKW, Driver and Kuniyuki (1984) medium for Paradox walnut; BA, N^6-benzyladenine; 2iP, N^6-(2-isopentenyl) adenine; IBA, indole-3-butyric acid

Introduction

Successful micropropagation has been reported for both embryonic and juvenile material of hazelnut (Anderson, 1984; Perez et al., 1987). However, culture of explants from mature tissue is limited by contamination and low multiplication rates (Bassil et al., 1992; Diaz-Sala et al., 1990; Messeguer and Mele, 1987). Several media formulations are used for hazelnut such as Anderson (Anderson, 1984), K(h) (Cheng, 1975) and several modified MS (Murashige and Skoog, 1962) media; sucrose has been used exclusively as the carbon source (Anderson, 1984; Bassil et al., 1992; Diaz-Sala et al., 1990; Messeguer and Mele, 1987; Perez et al., 1987). Some special mineral nutrient media developed for other woody plants have not been tested on hazelnut (Driver and Kuniyuki, 1984; Lloyd and McCown, 1981; Yang et al., 1986). Alternative carbon sources are used for the culture of *Alnus* (glucose), and *Malus* (sorbitol and fructose) and greatly improve growth and multiplication compared to sucrose (Welander et al., 1989). Whether sucrose is the best carbon source for hazelnut has not been determined.

This study was designed to optimize the culture medium for in vitro shoot multiplication and elongation of cultures derived from mature field-grown hazelnut trees.

Materials and Methods

Hazelnut cvs. Tonda Gentile Romana and Nonpareil from the in vitro collection at the National Clonal Germplasm Repository were used in this study. 'Tonda Gentile Romana' was received as in vitro material from Italy. 'Nonpareil' shoots were collected from mature trees, washed with soapy water and rinsed for 10 min with tap water, then shaken with 10% Clorox (1991 Clorox company, Oakland, CA) bleach (5% sodium hypochlorite) plus Tween 20 (Sigma) for 10 min followed by 2-3 rinses in sterile deionized water and cultured on MWPMC with 1 mg/l BA. Shoot tips and nodal segments from 1-2 year old in vitro cultures were used as explants in the experiments.

All media contained BA (1.5 mg/l) and IBA (0.01 mg/l), except for the cytokinin study. Media were solidified with 0.6% (w/v) agar (Difco Bitek, Detroit, MI) and autoclaved at 121°C for 20 min.

Growth room conditions were a 16-h photoperiod (25 μmol • m^{-2}• s^{-1}) at 25°C. Each treatment consisted of two Magenta GA7 boxes (Magenta, Chicago, IL), with 12 explants in each box. Experiments were done twice. Shoot number and length were measured after four weeks incubation. The final data were reported as the mean of four replications, with 48 total explants for each treatment, analyzed by analysis of variance and student's t-test, using Statgraphics software ($P \leq 0.05$).

Basal media. Three media, each with 3% sucrose and at pH 5.2, were compared: MWPMC (Yang et al., 1986) altered by substituting Sequestrene 138 Fe (Geigy) for FeDTPA; DKW medium (Driver and Kuniyuki, 1984) altered by

substituting agar for gelrite; and Anderson medium (Anderson, 1984) (Table 2.1).

Carbon source. Carbon sources (fructose, galactose, glucose, lactose, and sucrose) were tested on MWPMC at 3% (w/v) and on DKW at 3 and 5% (w/v). Additional glucose concentrations were tested at 1, 2, and 3% (w/v) on DKW.

pH of medium. Explants were compared on DKW with 3% sucrose at pH 4.7, 5.2, and 5.7.

Cytokinin. Experiment A. Four cytokinins (BA, zeatin, kinetin, or 2iP at 1.5 mg/l) were tested in combination with 0.01 mg/l IBA. The combination of 2 mg/l BA and 1 mg/l 2iP was also evaluated. The basal medium was DKW with 3% sucrose and at pH 5.2. *Experiment B.* Five concentrations of BA (0.5, 1, 1.5, 2, and 5 mg/l) and 12 combinations of BA (0, 1, 2, and 3 mg/l) and 2iP (0, 1, and 2 mg/l) were tested on DKW medium with 3% glucose and at pH 5.2.

Results and Discussion

Basal medium

Both shoot multiplication and elongation of the two cultivars differed significantly with the mineral nutrient medium used. DKW produced the most shoots (2.2 shoots/explant for 'Nonpareil' and 2.1 for 'Tonda Gentile Romana') but shorter shoots than did MWPMC. Proliferation on MWPMC (1.3 shoots/explant for 'Nonpareil' and 1.4 for 'Tonda Gentile Romana') was significantly lower than on DKW but higher than on Anderson medium. Only one shoot per explant was produced on Anderson medium; moreover, these shoots were usually necrotic. The results for mature shoots on Anderson medium in this study are in contrast with results when the medium was used with seedling material (Anderson, 1984), where Anderson medium was the most consistent and gave a higher shoot multiplication rate than did MS and woody plant medium (Lloyd and McCown, 1981). DKW, with high ionic and vitamin concentrations, is effective in promoting multiple shoot development for Paradox walnut (Driver and Kuniyuki, 1984); our results indicated that it also supported optimum multiple shoot development for hazelnut. MWPMC, with high Ca and Mg concentrations but with low N, P, and K concentrations, produces good results for Chinese chestnut (Yang et al., 1986) but is not optimal for hazelnut.

Carbon source

Explants on MWPMC with 3% fructose or lactose produced more shoots than those on glucose or sucrose, although differences between fructose, glucose, and sucrose generally were not significant (Table 2.2).

Shoots grown on fructose, sucrose, and glucose were similar in shoot length and leaf color, but those on lactose were shorter and greener. On galactose, 60% of the explants turned brown and died; those remaining produced one short shoot each.

On DKW medium, significant differences were observed in both mean shoot number and mean shoot length between the five carbon sources tested at 3% concentrations (Table 2.3).

For both cultivars, explants on glucose, lactose, and fructose produced the most shoots. Although more than two shoots were produced per explant on sucrose, the multiplication rate was significantly lower than on glucose, lactose, or fructose. Explants grown on galactose had high mortality rates (mean 71%) resulting from explant browning. Significantly fewer shoots were produced on galactose than on the other sugars. This is similar to the effect of sorbitol on *Syringa*, where death of explants was attributed to lack of translocation by the sieve-tubes (Welander et al., 1989).

The best shoot elongation of 'Tonda Gentile Romana' was on fructose and glucose, and significantly longer shoots of 'Nonpareil' were obtained on fructose than the other carbon sources (Table 2.3). Regulation of multiplication in vitro by carbon

source is also documented by Pua and Chong (1985) and Welander et al. (1989), where the best carbon source is glucose for *Alnus* and fructose for *Malus* (Welander et al., 1989). Although sucrose is the most used carbon source in culture media and is commonly used for hazelnut, our results indicate that improvements in multiplication rate and explant size can be realized by using glucose or fructose.

Significantly higher rates of shoot proliferation were obtained with all five carbon sources at 3% than at 5% (data not shown). Callus was produced at the base of shoots grown on 5% fructose. The general appearance and growth habit of shoots were better on medium with glucose than fructose. Therefore, glucose was chosen for further studies.

In order to optimize the glucose concentration in the medium, three levels (1, 2, and 3%) were tested with DKW as the basal medium. Explants on 3% glucose produced the largest number and longest shoots, but means were not significantly greater than for the 2% level. Those on 1% glucose were significantly shorter and produced fewer shoots than on either 2 or 3% (Fig. 2.1).

pH

Shoot number and length were not significantly different among explants grown on DKW at pH 4.7, 5.2, and 5.7 (data not shown). Hazelnut cultures are thus adaptable to a range of acidity similar to blueberry, which can grow on Lloyd and McCown's woody plant medium with a pH range from 4.0 to 6.0 (Wolfe et al., 1986).

Cytokinin

Experiment A: BA or zeatin (1.5 mg/l) and the combination of BA (2 mg/l) with 2iP (1 mg/l) produced more shoots than kinetin or 2iP at 1.5 mg/l (data not shown). Explants on kinetin or 2iP at 1.5 mg/l did not produce new shoots, or produced only one shoot per explant. Our results are in agreement with those obtained by Messeguer and Mele (1987) who found that kinetin and 2iP had fewer promotive effects than did BA in producing shoots from hazelnut embryos. Anderson (1984) also found that kinetin was ineffective for hazelnut shoot multiplication, but BA produced healthy shoots and some multiplication.

Experiment B: A comparison of BA concentrations and combinations of BA and 2iP indicated that there were significant differences between BA concentrations, but no significant differences for 2iP concentrations from 0 to 2 mg/l (Table 2.4). The addition of 2iP did not alter the effectiveness of BA and both cultivars had a similar pattern of response to cytokinins. Our results are in contrast with Anderson's observation for seedling material that the combination of BA (2 mg/l) with 2iP (1 - 2 mg/l) was the best for shoot multiplication. The suitable BA concentrations in our experiments were in the range of 1.5 - 3 mg/l.

Genotype

Elongation of explants varied between the two cultivars. 'Nonpareil' shoots were taller than those of 'Tonda Gentile Romana' (on DKW with BA at 1.5 mg/l,

IBA at 0.01 mg/l and 3% glucose). Both cultivars produced similar numbers of shoots (Table 2.3). Shoots of 'Barcelona', 'Riccia di Talanico', 'Ruby', 'OSU 20-58', 'Palaz', 'Ennis', 'Giresun 54-41', 'USOR 1-70', 'USOR 2-72', 'Burchardt's Zellernuss', and 'Willamette' were grown on the best medium tested with good results (data not shown). We have found differences in shoot multiplication and elongation among hazelnut cultivars similar to that reported by Bassil et al. (1992).

Conclusions

Several improvements were determined for multiplication of explants of mature hazelnut in vitro. The multiplication rate of hazelnut cvs. Nonpareil and Tonda Gentile Romana was affected by the mineral composition of the medium, carbon source and concentration, cytokinin, and genotype, but not by changes in pH. For optimal hazelnut shoot multiplication, elongation and appearance, we recommend the use of a modified DKW medium with 3% glucose, BA (1.5 - 3 mg/l) and IBA (0.01 mg/l), with subculture at 4-week intervals. Shoots of more than 10 genotypes multiplied well on this medium. These improvements will provide a basis for further research on hazelnut micropropagation.

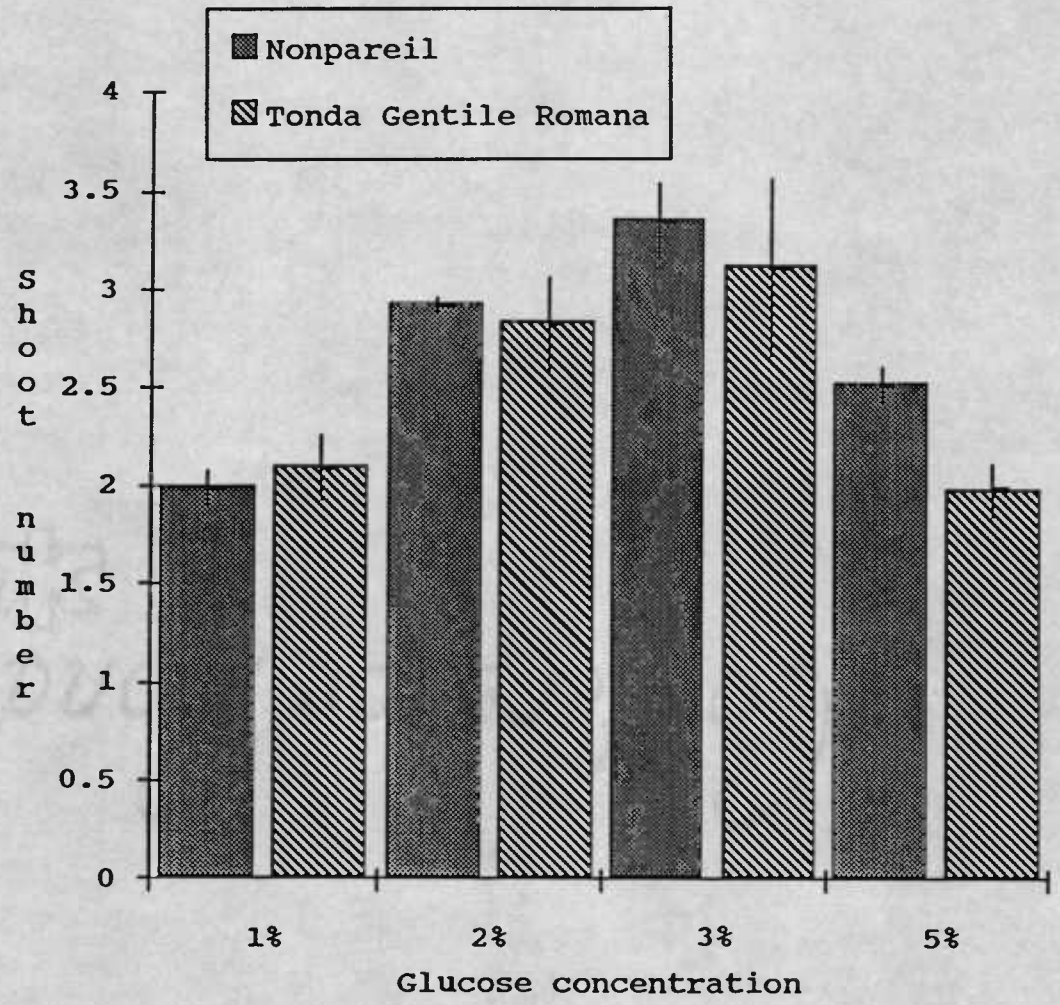

Fig. 2.1. Effect of glucose concentration on the multiplication of hazelnut shoot cultures (pooled data from two experiments).

Table 2.1. Components (mg/l) of DKW medium, MWPMC and Anderson media.*

Component	DKW	MWPMC	Anderson
$CaNO_3 \cdot 4H_2O$	1960	1112	0
KNO_3	0	0	480
$NH_4 \cdot NO_3$	1417	400	400
$Zn(NO_3)_2 \cdot 6H_2O$	17	0	0
$ZnSO_4 \cdot 7H2O$	0	8.6	8.6
K_2SO_4	1559.5	990	0
$MgSO_4 \cdot 7H_2O$	740	740	370
$MnSO_4 \cdot H_2O$	33.8	16.9	16.9
$CuSO_4 \cdot 5H_2O$	0.25	0.025	0.025
$CaCl_2 \cdot 2H_2O$	147	192	440
KH_2PO_4	258.4	170	0
$NaH_2PO_4 \cdot H_2O$	0	0	380
H_3BO_3	4.8	6.2	6.2
$NaMoO_4 \cdot 2H_2O$	0.39	0.25	0.25
KI	0	0	0.30
$CoCl_2.6H_2O$	0	0	0.025
Na_2EDTA	45	0	74
$FeSO_4 \cdot 7H_2O$	33.4	0	55.7
Sequestrene 138 Fe	0	200	0
Thiamine	2	1	0.4
Myo-inositol	1000**	100	100
Glycine	2	2	0
Nicotinic acid	1	0.5	0
Adenine Sulfate	0	0	80
Sucrose	30000	30000	30000

* DKW (Driver and Kuniyuki, 1984); MWPMC (Yang et al., 1986); Anderson (Anderson, 1984).

** A misprint in the original paper; 100 mg/l is correct.

Table 2.2. Effect of carbon source on shoot multiplication of *C. avellana* cvs. Nonpareil and Tonda Gentile Romana (T.G.R.) after four weeks of culture on MWPMC medium.

Carbon source (3%)	Shoot multiplication[A]		Shoot length (cm)[B]	
	Nonpareil	T. G. R.	Nonpareil	T. G. R.
Glucose	1.4b	1.3ab	2.2a	2.8a
Fructose	1.6ab	1.7a	2.3a	2.4b
Lactose	1.9a	1.6a	1.6b	1.4c
Sucrose	1.3bc	1.3ab	2.4a	2.3b
Galactose	1.0c	1.0b	1.1c	0.7d

[A] Mean number of shoots formed per explant.
[B] Mean length of new shoots only.
Values followed by the same letter in a column are not significantly different at P = 0.05.

Table 2.3. Effect of carbon source on shoot multiplication of *C. avellana* cvs. Nonpareil and Tonda Gentile Romana (T.G.R.) after four weeks of culture on DKW medium.

Carbon source (3%)	Shoot multiplication[A]		Shoot length (cm)[B]	
	Nonpareil	T. G. R.	Nonpareil	T. G. R.
Glucose	3.4a	3.1a	1.7b	1.3a
Fructose	3.4a	3.0ab	2.0a	1.4a
Lactose	3.3a	3.2a	1.2c	0.9b
Sucrose	2.2b	2.8b	1.6b	0.9b
Galactose	1.4c	1.7c	1.0d	0.9b

[A] Mean number of shoots formed per explant.
[B] Mean length of new shoots only.
Values followed by the same letter in a column are not significantly different at P = 0.05.

Table 2.4. Effect of BA and 2iP on shoot multiplication of *C. avellana* cvs. Nonpareil and Tonda Gentile Romana (T.G.R.) after four weeks of culture on DKW medium.

Cytokinin (mg/L)		Shoot multiplication[A]		
BA	2iP	Nonpareil	T. G. R.	Mean of BA[B]
0	0	0.19	0.17	0.22a
	1	0.38	0.21	
	2	0.29	0.08	
1	0	2.86	2.74	2.84b
	1	2.92	2.92	
	2	2.79	2.81	
2	0	3.11	3.05	3.13c
	1	2.96	3.29	
	2	3.15	3.2	
3	0	3.20	3.13	3.02bc
	1	3.11	3.02	
	2	2.85	2.79	

[A] Mean number of shoots formed per explant.
[B] Mean number of shoots at different BA concentrations. Values followed by the same letter are not significantly different at P = 0.05.

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Chapter 3

A MICROPROPAGATION SYSTEM FOR HAZELNUTS (*CORYLUS* SPECIES)

Abstract

A micropropagation system was developed for hazelnut cultivars. Explants collected from grafted greenhouse-grown plants had a high percentage of growing explants and little tissue contamination or oxidation. For mature field-grown trees, explants from suckers in July had a higher percentage of growing explants than those from other field materials. Tissue contamination and oxidation were the main problems for in vitro establishment of mature field-grown trees. Explants collected from grafted greenhouse-grown plants in March had a higher initiation frequency than those collected later. Multiplication of shoots was similar with either 1.5 mg•liter^{-1} BA with or without 0.01 mg•liter^{-1} IBA; or 2 mg•liter^{-1} BA with 1 mg•liter^{-1} 2iP, and all three were better than with 5 mg•liter^{-1} BA. Three to five new explants were produced from one original explant after four weeks of culture on NCGR-COR medium supplemented with 1.5 mg•liter^{-1} BA and 0.01 mg•liter^{-1} IBA. Roots were produced on 64% to 100% of shoots grown on half strength NCGR-COR mineral salts and 1 mg•liter^{-1} IBA for four weeks. Ex-vitro rooting of shoots by a brief dip in 0.2 or 1 g•liter^{-1} IBA was equally successful. Transplant survival of plants was 78% to 100%. Chemical names used: N^6-benzyladenine (BA); indole-3-acetic acid (IAA); indole-3-butyric acid (IBA); N^6-(2-isopentenyl)adenine (2iP).

Introduction

Historically, in many hazelnut (*Corylus*) growing regions of the world, cultivars have been propagated as self-rooted trees by layerage (Lagerstedt, 1992). Layered trees continually produce suckers which require periodic control. Grafting may be a more efficient propagation technique, but would be feasible on a commercial scale only with sufficient suitable rootstocks. A micropropagation system could be an alternative to traditional field layering propagation for fast dissemination of new hazelnut cultivars. Micropropagation of hazelnut has been demonstrated with seedling and juvenile materials but reports of successful culture with adult materials are scarce because of the problems of microbial contamination and low multiplication rate (Messeguer and Mele, 1987; Perez et al., 1987; Diaz-Sala et al., 1990; Bassil et al., 1992). Recent improvements in hazelnut micropropagation have provided increased opportunities for micropropagation of hazelnut cultivars (Bassil et al., 1992; Diaz-Sala et al., 1990; Yu and Reed, 1993).

The explant source is one of the most important factors that affect the in vitro response. Juvenile materials are generally easier to propagate than mature ones; therefore, selection of the most juvenile tissues within a tree and/or rejuvenation of parts of the donor tree by special treatments prior to excision of explants have been commonly adopted in woody plants (Bonga, 1987). Root suckers and stump sprouts are more juvenile than other branches and thus provide a good choice for explants (Thorpe and Harry, 1990). Rejuvenation or reinvigoration techniques include repeated spraying of branches intended for explant with cytokinin (commonly N^6-

benzyladenine, BA) (Abo El-Nil, 1982), serial grafting (Franclet et al., 1987), repeated grafting of a scion from a mature tree onto seedling rootstocks, serial rooting of mature cuttings, and repeated subculture of shoot apices in cytokinin-containing medium (Thorpe and Harry, 1990).

Hazelnut micropropagation has been described using rejuvenated or reinvigorated adult tissues. Messeguer and Mele (1987) used rooted branches of mature plants of hazelnut cv. Gironell, grown for 2 years in the greenhouse but obtained less than 50% growth of explants. Perez et al. (1987) used lateral buds taken from the vegetative shoots of 12-month old greenhouse-grown cuttings of cv. Negret and obtained 70% active growth of buds in vitro. Diaz-Sala et al. (1990) obtained 80% bud outgrowth of explants from forced shoots of cold-stored field-grown mature branches of cv. Tonda Gentile delle Langhe. In addition to the source of explants, the season in which explants are collected also influences in vitro response. For example, the highest percentage of growing explants was obtained from greenhouse-grown plants of *Corylus avellana* cv. Negret collected in the autumn (Messeguer and Mele, 1983).

The objective of this study was to develop a micropropagation system for hazelnut rootstock and nut cultivars.

Materials and Methods

Six- to 10-year-old trees of *Corylus avellana* cvs. Barcelona, Gasaway, Willamette, Dundee, and Newberg were used as material sources. 'Barcelona' is the leading cultivar in Oregon, 'Gasaway' is a pollinizer and is highly resistant to Eastern filbert blight (Mehlenbacher et al., 1991b), and 'Willamette' is a new cultivar for the blanched kernel market (Mehlenbacher et al., 1991a). 'Newberg' (USOR 7-71) and 'Dundee' (USOR 15-71) are two new non-suckering rootstocks, selected from open-pollinated *C. columa* seed. Based on morphological characterics, they appear to be *Corylus* interspecific hybrids (Lagerstedt, 1990 and 1993). 'Barcelona' (NCGR accession # 36) and 'Gasaway' (NCGR accession # 54), 'Dundee' (NCGR accession # 165), and 'Newberg' (NCGR accession # 168), were taken from collections of the National Clonal Germplasm Repository (NCGR) at Corvallis, Oregon and 'Willamette' was collected from the Oregon State University, Department of Horticulture Research Farm.

Culture establishment. Preliminary experiments indicated that grafting on seedling rootstocks had beneficial effects on hazelnut culture establishment. Therefore we confirmed the experiment with explants of 'Barcelona', and 'Gasaway' collected from the following sources:

1). Grafted greenhouse-grown plants: Dormant branches were collected from field-grown trees in December, cold stored (4 °C) and grafted onto seedling rootstocks ('Barcelona') in a greenhouse in January using whip and tongue grafting. The grafted plants grew in the greenhouse for the rest of growing season.

2). Upper branches of field-grown trees.

3). Suckers of field-grown trees.

Explants from grafted greenhouse-grown plants were collected in March, May, and July, those from upper branches in May, June, and August, and those from suckers in June, July, and September.

Forced outgrowth of field-grown branches of 'Gasaway' were also tried. Field-grown branches were collected in April, washed with tap water and pre-sterilized with 15% bleach (containing 5.25% sodium hypochlorite, Clorox, Oakland, CA) solution with a few drops of Tween 20 (polyoxyethylene sorbitan monolaurate, Sigma, St. Louis, MO) for 10 minutes, and rinsed with tap water. After the bases were recut they were immersed in forcing solution (9 g/l floralife, Floralife, Inc. Burr Ridge, IL) at 23 $\pm$ 2 °C in the laboratory. The basal end of branches were pruned off and the solutions replaced weekly. New shoots were collected after 3-4 weeks (in May).

Three new cultivars, 'Willamette', 'Dundee', and 'Newberg', were used to determine whether grafted greenhouse-grown plants of varied genotypes produced a high rate of culture establishment. Explants from 'Willamette', 'Dundee', and 'Newberg' were taken from grafted greenhouse-grown plants (as described above) in March, May, and July.

Shoots were collected, washed with soapy water and rinsed with tap water after the leaves were trimmed off. Shoots then were cut into single-node segments and surface sterilized in 15% bleach solution with a few drops (one drop per 80 ml) of Tween 20 for 10 minutes followed by two rinses with sterile deionized water. Only

lateral-bud segments were used for most experiments. Shoot tips from grafted greenhouse-grown plants of 'Barcelona', 'Gasaway' and 'Willamette' in March were also used to compare the effects of apical shoot tips and nodal segments. The explants were recut into segments of 1-1.5 cm long and planted individually in 16 x 100 mm culture tubes containing five ml culture medium. Fifteen explants were used per experiment.

The culture medium used was NCGR-COR medium, a modified DKW medium (Driver and Kuniyuki, 1984) altered by substituting glucose for sucrose, 200 mg per liter of Sequestrene 138 Fe (Geigy) for FeEDTA, and 5 g per liter of agar (Difco Bitek, Detroit, MI) for Gelrite, with 5 $mg \cdot l^{-1}$ N^6-benzyladenine (BA) and 0.01 $mg \cdot l^{-1}$ indole-3-acetic acid (IAA). Cultures were placed in a growth room at 25°C, with a 16 h photoperiod and 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ cool-white fluorescent illumination. After four weeks in culture, percent contaminated, oxidized, and proliferated (budbreak and elongation) explants (based on total explant number) were determined and new shoots were subcultured.

To decrease tissue oxidation, explants from upper branches and suckers of field-grown trees were treated in three ways: 1) immersed in a solution (per liter of water) of 1 g ascorbic acid for 2 h, or 24 mg cysteine for 1 h, or 1 g 8-hydroxy-quinoline hemisulfate salt (8-HQS) (Sigma, St. Louis, MO) for 8 h following cleaning with soapy water but before surface sterilization; 2) immersed in a solution (per liter of water) of 2 g diethyldithiocarbamic acid, sodium salt (DIECA) solution (filter sterilized) for 2 h or 200 mg $K_2S_2O_7$ (filter sterilized) for 1 min immediately after surface sterilization, and 3) covered with 0.1 % solution of 8-HQS (filter sterilized)

applied to the top of agar medium for the first 6, 12, or 24 h of culture on medium and then transferred onto fresh culture medium (Laimer da Camara Machado et al., 1991).

In order to determine the relationship between phenolic content and tissue oxidation, shoot tips and nodal segments (0.2 g) from grafted greenhouse-grown plants and upper branches and suckers of field-grown trees of hazelnut cvs. Gasaway and Barcelona in June were used to analyze phenolic content. Methods described by Oydvin and Richardson (1987) were used as follows:

1). Extraction of phenolics from tissue. Samples were treated with 10 ml 80% ethanol and cut into small pieces, ground in a mortar, and filtered through bleached absorbent cotton into test tubes. The samples were evaporated in nitrogen flow to one fifth the original volume before use.

2). Paper chromatography. 100 μl of the 5x extract was spotted on paper (Whatman No. 3 MM paper) and phenolics were separated by two-dimensional paper chromatography. Solvents used for the first dimension were secondary butylalcohol, glacial acetic acid, and water (70 : 2 : 28) and for the second dimension 2% glacial acetic acid in water (2% v/v).

3). Detection of spots on paper chromatogram. All chromatograms were examined in a dark room under u. v. light (366 w. l.) both with and without ammonia-fuming. Detected spots were rated visually for color intensity.

Shoot multiplication. Shoots from established cultures of 'Willamette', 'Dundee', and 'Newberg' were cut into nodal segments and shoot tips, grown in Magenta GA7 boxes (Magenta, Chicago, IL) containing 40 ml NCGR-COR medium

supplemented with 1.5 mg•liter^{-1} BA and 0.01 mg•liter^{-1} indole-3-butyric acid (IBA) and subcultured at four week intervals. Growth regulator combinations (per liter) of 1.5 mg BA with or without 0.01 mg IBA, 5 mg BA, and 2 mg BA with 1 mg N6-(2-isopentenyl)adenine (2iP) were compared for shoot multiplication with 24 explants per treatment and two replications for each genotype. Data for shoot number produced per explant and average shoot length were collected after four weeks of culture and analyzed.

Root induction and acclimatization. Root induction was first tried in-vitro. Shoots (2-3 cm long) formed on multiplication medium were transferred to Magenta GA7 boxes containing NCGR-COR medium at half of the normal concentration of mineral salts with 1 mg•liter^{-1} IBA for four weeks. Both rooted and unrooted shoots were transplanted into 2-inch plastic pots containing a mix of vermiculite and perlite (1 : 1), placed in a mist bed for two to three weeks, then on the greenhouse bench for another two weeks and finally transferred to 4-inch pots with a standard greenhouse mix.

Ex-vitro root induction of in-vitro shoots was then carried out. The bases of 3-4 cm long shoots were dipped in 0, 0.2, or 1 g•liter^{-1} IBA solution (dissolved in a small amount of 95% alcohol then brought to final volume with deionized water) for one min and planted in plastic pots as described above.

Fourteen shoots for each rooting treatment and cultivar and two replications were performed for root induction and acclimatization.

Results and Discussion

Culture establishment

The initial responses of explants were categorized as tissue oxidation (explant browning) and microbial contamination (bacteria and fungi), bud growth, and no growth. Tissue oxidation and contamination of explants were evident after 5 - 7 days of culture. Tissue oxidation was confined to the plant tissue and did not stain the medium. Healthy explants started to show budbreak and elongation (bud growth) in 7 - 10 days and new shoots were subcultured after four weeks. Some explants did not show contamination, tissue oxidation, or bud growth after four weeks of culture (no response). After four weeks of culture, the percentage of these explant responses differed among explant source and explant collecting season (Tables 3.1 and 3.2).

For cultivars Barcelona and Gasaway, there were differences in the percentage of growing explants among the explant sources and explant collecting seasons (Table 3.1). Overall, grafted greenhouse-grown plants were the best explant source. Explants from the grafted greenhouse-grown plants collected in March had the highest percentage of growing explants and very low tissue contamination and oxidation. Grafting has not previously been used as a method of pretreatment for hazelnut tissue culture. Explants from suckers collected in July, those from 'Barcelona' grafted plants in May, and those from 'Gasaway' grafted plants in July had lower but acceptable levels of explant growth. Explants from upper branches in all seasons had the lowest percentage of explant growth and high tissue

contamination and oxidation. Explants from forced outgrowth of upper branches of field-grown trees of 'Gasaway' were used to determine whether we could eliminate some tissue contamination by forced outgrowth, but the percentage of growing explants (19%) remained the same as for upper branches. This is similar to the results of Diaz-Sala et al. (1990) who reported that 80% of single buds of forced mature branches without cold treatment of hazelnut 'Tonda Gentile delle Langhe' failed to respond and the remaining 20% showed only leaf expansion and slight shoot elongation and did not develop further.

Differences between nodal segments and shoot tips were also observed with nodal segments better than shoot tips. Shoot tips initially showed leaf expansion but soon died with none developing further. Messeguer and Mele (1987) reported that shoot tips of hazelnut 'Negret' died after a few days in culture. We also noted a similar problem with shoot tips of forced shoots (data not shown). It is probable that there are some physiological differences between shoot tips and nodal segments that are causing this phenomenon.

Tissue oxidation in hazelnut, like some other woody plants, is a serious problem during culture establishment. Several approaches have been developed to deal with this problem in woody plants (Preece and Compton, 1991). In the present study, treatment of explants from field-grown trees with solutions of ascorbic acid, cysteine, 8-HQS, DIECA, or $K_2S_2O_7$ did not decrease tissue oxidation (data not shown). The best method for avoiding tissue oxidation remains the use of explants from grafted plants (Table 3.1 and 3.2).

Paper chromatography indicated that tissues from field-grown trees had more phenolic compounds than tissues from grafted greenhouse-grown plants and shoot tips had more than nodal segments (Data not shown). These results substantiate our observations from in-vitro culture establishment that explants from grafted greenhouse-grown plants showed little tissue oxidation while it was very severe in explants from field-grown trees (Table 3.1). A similar relationship was noted with nodal segments and shoot tips. Yu and Meredith (1986) also reported that axillary shoots of grape (*Vitis vinifera*) had greater survival and less endogenous phenolic content than terminal shoots.

Effects of grafted greenhouse-grown plants on culture establishment were further confirmed in the three new hazelnut cultivars Willamette, Dundee, and Newberg. All explants collected in March had high percentages of explant growth and were better than those collected later, and tissue contamination and oxidation were acceptably low (Table 3.2 and Fig. 3.1).

Based on these results we conclude that grafted greenhouse-grown plants collected in March are the best explant source and suckers collected in July would be the best option for field collection of materials for culture establishment. Stock (mother) plants in the greenhouse have been used to decrease tissue contamination (Messeguer and Mele, 1983) and juvenile materials have been reported to have better growth response and less oxidation than mature materials in conifers (Kurz, 1986). Our results show that grafted greenhouse-grown hazelnut plants may be quickly rejuvenated or reinvigorated and protected from bacterial contamination, thus providing an excellent explant source in a short period of time. Messeguer and Mele

(1983) also found that the collecting season influenced explant response but their best results (63% of explants sprouted) with greenhouse-grown plants of 'Negret' were those collected in the autumn. The differences between our results and Messeguer's may reflect differences in genotype as well as the difference between grafted and self-rooted plants.

Shoot multiplication

Optimum medium for hazelnut shoot multiplication was reported previously (Yu and Reed, 1993). The three new hazelnut cultivars tested in the present study had responses similar to our earlier results with *C. avellana* cvs. Nonpareil and Tonda Gentile Romana (Yu and Reed, 1993). BA at 5 mg•liter^{-1} produced significantly ($P \leq 0.05$) fewer and shorter shoots than all the other combinations of growth regulators tested for all three cultivars (Data not shown). The longest shoots were produced on medium with the combination of 1.5 mg•liter^{-1} BA and 0.01 mg•liter^{-1} IBA but were not significantly longer than those with 1.5 mg•liter^{-1} BA alone. The combination of 1.5 mg•liter^{-1} BA and 0.01 mg•liter^{-1} IBA was used for shoot multiplication based on overall plant appearance. After two to three subcultures on this medium, single shoots were cut into shoot tip and nodal segments with single buds (explant) and each explant produced one to two new shoots (Fig. 3.2). Of the two shoots produced, one was usually 2-4 cm long and was divided into three new explants; the other (0.5-1 cm) produced one or two new explants, resulting in a total of three to five new explants per original explant in 4 weeks. Shoot number

produced per explant was not different among three cultivars (Data not shown). Shoots of 'Dundee' were significantly longer than those of 'Willamette' while shoots of 'Newberg' were intermediate in length. Internal bacterial contamination was observed after a few subcultures but did not kill the plants.

Root induction and acclimatization

Rooting frequency varied among the three cultivars. After four weeks on rooting medium, the rooting frequencies were 64% for 'Willamette', 78% for 'Newberg', and 100% for 'Dundee'. More than four 2-5 cm long roots were obtained per shoot for each of the three cultivars. Both rooted and unrooted shoots were transplanted to the greenhouse without further treatment and all of them rooted and survived after four weeks of acclimatization.

Since a high survival rate was achieved after acclimatization for both rooted and unrooted shoots and roots were obtained from unrooted shoots cultured in rooting medium, we tried ex-vitro root induction and acclimatization. Ex-vitro rooting occurred in two to three weeks and high rooting frequencies were obtained with dipping in 0.2 or 1 g•liter^{-1} IBA solution (Table 3.3). More (5-10 roots/shoot) and shorter (1-3 cm long) roots were produced with 1 g•liter^{-1} IBA than with 0.2 g•liter^{-1} IBA (about 5 roots/shoot and 2-5 cm long). Shoots dipped in water (0 g•liter^{-1} IBA) required a longer time to produce roots (about six weeks) and had a lower rooting frequency and fewer roots than shoots dipped in the IBA solutions. For 0.2 or 1 g•liter^{-1} IBA dip treatments, 'Dundee' and 'Willamette' rooted better

than 'Newberg'. For 0 g•liter^{-1} IBA dip treatment, 'Willamette' rooted the best and 'Newberg' the worst.

For both in-vitro and ex-vitro rooting studies, plants were transplanted after loosening the lids of Magenta GA7 boxes in the culture room for one or two days. The plants first withered but recovered after they were transplanted. High survival rates (78% to 100%) were achieved for both in-vitro and ex-vitro root induction of all three cultivars, but 'Newberg' had a lower survival rate than the other two cultivars and those with 1 g•liter^{-1} IBA dip treatment had a lower survival rate than those with other rooting treatments (Table 3.3). Hazelnuts may develop dormancy after greenhouse establishment and may require a period of cold temperature to break dormancy. After the plantlets started to grow, they grew rapidly (Fig. 3.3).

This is the first report of ex-vitro rooting of micropropagated hazelnut shoots. In-vitro rooting is a time and labor consuming process and based on our experience we suggest using ex-vitro rooting to save time, labor, and money.

Conclusions

Micropropagation of hazelnut was achieved by selecting explants (nodal axillary segments) from shoots of grafted greenhouse-grown plants in March. NCGR-COR medium with 5 mg•liter^{-1} BA and 0.01 mg•liter^{-1} IAA was used for culture establishment and 1.5 mg•liter^{-1} BA and 0.01 mg•liter^{-1} IBA for shoot multiplication. High frequencies of rooting were induced either in vitro on medium with 1 mg•liter^{-1} IBA or ex vitro by dipping in 0.2 or 1 g•liter^{-1} IBA for one min then acclimatization. A high acclimatization survival rate was achieved by growing plants in a mixture of vermiculite and perlite (1 : 1) under mist for two to three weeks and then transferring to the greenhouse bench.

Table 3.1. Effects of genotype, explant source, and collecting season on the growth, contamination, and oxidation of hazelnut explants after four weeks of culture on NCGR-COR medium with 5 mg•liter^{-1} BA and 0.01 mg•liter^{-1} IAA.[a]

Genotype	Explant source	Explanting month	Percentage explants			
			Growing	Cont.	Oxidized	No response
Barcelona	Grafts	March	67	13	0	20
		May	46	0	33	20
		July	27	0	60	13
	Upper branches	May	13	60	33	0
		June	0	13	74	13
		August	0	93	7	0
	Suckers	June	13	20	40	27
		July	47	47	13	7
		Sept.	6	40	46	7
Gasaway	Grafts	March	87	0	0	13
		May	27	0	7	67
		July	60	7	7	33
	Upper branches	May	13	67	27	0
		June	7	7	27	60
		August	0	47	27	27
	Suckers	June	27	40	40	13
		July	60	20	20	7
		Sept.	0	7	80	13

[a] Growing = (No. of explant showing bud break and elongation/No. of total explants) x 100

Cont. = (No. of explant contaminated/No. of total explants) x 100

Oxidized = (No. of explant browning/No. of total explants) x 100

No response = (No. of total explants - No. of bud breaking and elongation - No. of explant contamination and oxidation)/No. of total explant x 100

Table 3.2. Effects of genotype and explant collecting season on the growth, contamination and oxidation of hazelnut explants after four weeks of culture on NCGR-COR medium with 5 mg•liter^{-1} BA and 0.01 mg•liter^{-1} IAA.

Genotype	Explanting month	Percentage of explants			
		Growing[z]	Cont.[y]	Oxidized[x]	No response[w]
Dundee	March	67	0	20	7
	May	27	7	0	67
	July	20	33	0	47
Newberg	March	100	0	0	0
	May	93	0	0	7
	July	47	13	7	40
Willamette	March	87	0	13	0
	May	53	0	27	20
	July	47	0	20	33

[z] Growing = (No. of explants showing budbreak and elongation/Total No. of explants) x 100

[y] Cont. (contaminated) = (No. of contaminated explants/Total No. of explants) x 100

[x] Oxidized = (No. of oxidized explants/Total No. of explants) x 100

[w] No response = (Total No. of explants - No. of explants showing budbreak and elongation - No. of contaminated and oxidized explants)/Total No. of explants x 100

[v] Values in a column followed by same letter are not significantly different ($P \leq 0.05$)

Table 3.3. Effects of ex-vitro rooting treatments on root induction and the survival rate of transplanted plantlets of hazelnut after four weeks of rooting and acclimatization in the greenhouse.

Treatment	Rooting frequency (%)			Acclimatization survival(%)		
	Newberg	Dundee	Willamette	Newberg	Dundee	Willamette
IBA 0 g/l	36	64	93	100	100	100
IBA 0.2 g/l	78	93	100	93	100	100
IBA 1 g/l	64	93	86	78	100	86

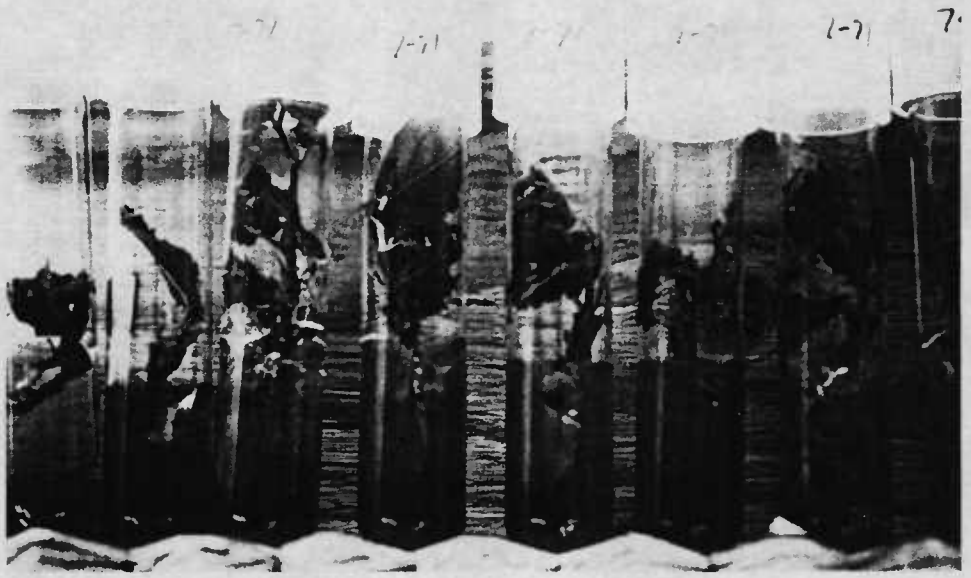

Fig. 3.1. Culture establishment from nodal segments of grafted greenhouse-grown plants of 'Newberg' in NCGR-COR medium with 5 $mg \cdot l^{-1}$ BA and 0.01 $mg \cdot l^{-1}$ IAA after growth for four weeks.

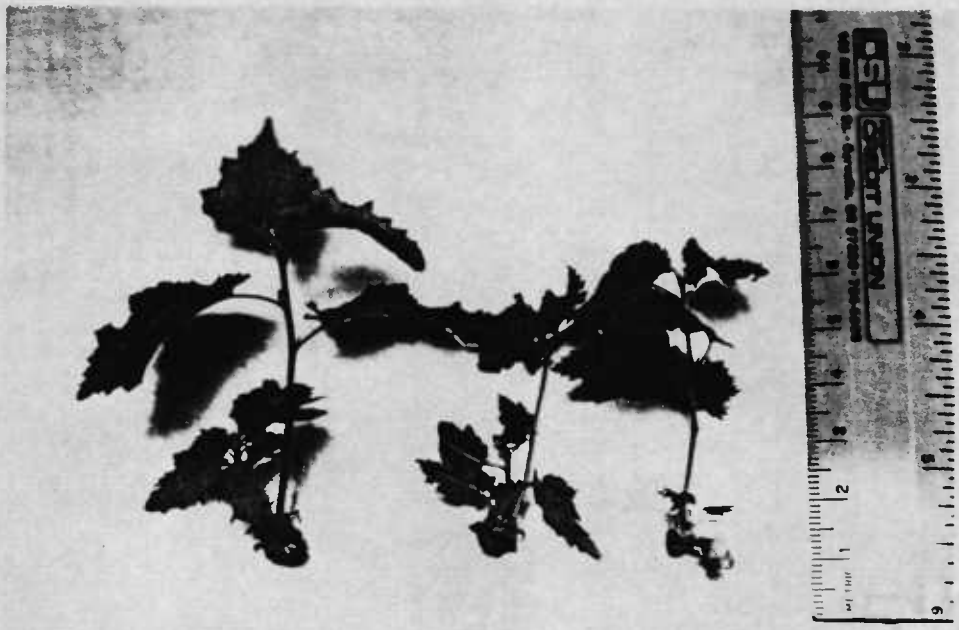

Fig. 3.2. Shoot multiplication of hazelnut rootstock cultivar Dundee after four weeks on NCGR-COR medium with 1.5 $mg \cdot l^{-1}$ BA and 0.01 $mg \cdot l^{-1}$ IBA.

Fig. 3.3. Hazelnut cultivar Willamette in-vitro plantlets established in the greenhouse by ex-vitro rooting (by dipping into 0.2 $g \cdot l^{-1}$ IBA for one minute) and acclimatization for two months.

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Chapter 4

EFFECTS OF ANTIBIOTICS ON INTERNAL BACTERIAL CONTAMINATION OF MICROPROPAGATED HAZELNUT

Abstract

Bacteria were isolated from contaminated shoot cultures of eight hazelnut genotypes (*Corylus avellana* L.). Both Gram-negative and Gram-positive forms with varied colony morphology were observed. Streptomycin, gentamicin, and rifampicin were bactericidal for individual bacterial isolates but none was effective for all the isolates. The bactericidal effect of antibiotics on bacterial isolates was different from that on bacteria established within plant tissues. A combination of 500 $\mu g \cdot ml^{-1}$ streptomycin and 15 $\mu g \cdot ml^{-1}$ gentamicin eliminated the internal bacterial contamination in 40-90% of shoot cultures of three *C. avellana* genotypes without severe phytotoxicity. Single antibiotics and a combination of 500 $\mu g \cdot ml^{-1}$ streptomycin and 15 $\mu g \cdot ml^{-1}$ rifampicin were ineffective. Phytotoxicity of antibiotics varied with types of antibiotics and plant genotypes.

Introduction

Commercial micropropagation laboratories very often report that persistent bacterial and fungal contamination is a serious problem (Boxus and Terzi, 1988; Kunneman and Faaij-Groenen, 1988; Cassells, 1990; Cornu and Michel, 1987). Failure of surface sterilization procedures to produce aseptic cultures is especially a problem with woody plants. Isolation of meristems (Hakkaart and Versluijs, 1983) and explants from stock plants grown under controlled conditions (Messeguer and Mele, 1983) have been used to obtain aseptic cultures. Contamination does not always show up at the culture establishment stage; some internal contaminants become evident at later subcultures and are difficult to eliminate (Thorpe and Harry, 1990). Antibiotics or other treatments may be needed to eliminate microbial contamination (Kneifel and Leonhardt, 1992; Leifert et al., 1991; Chevreau et al., 1989; Phillips et al., 1981), but the type and level of antibiotics and the duration of treatment useful for different plant tissue cultures vary and therefore need to be determined before use (Leifert et al., 1991; Cornu and Michel, 1987).

Internal bacterial contamination was observed in hazelnut shoot cultures in our laboratory. Contaminants were evident at culture establishment or became apparent after several subcultures of shoot multiplication. Loss of plants resulted when bacteria overgrew plant material but some explants survived and continued to grow with bacteria present. In this study we isolated bacteria from hazelnut shoot cultures and determined effects of antibiotic treatments on bacteria and plant materials to develop a procedure to eliminate bacterial contamination.

Materials and Methods

Shoot cultures of eight hazelnut accessions (*Corylus avellana* L.) from the USDA-ARS National Clonal Germplasm Repository (NCGR) collections were used: 'Nonpareil' (NCGR local accession number COR37), 'Tonda Gentile delle Langhe' (COR114), 'Tonda Gentile Romana' (COR475), 'Tonda Gentile delle Langhe' X 'Cosford sel. 3L' (COR494), 'Willamette' (COR500), 'Khachapura' (COR553), 'Quiros' (COR563), and 'Ros de la Selva' (COR565). They were established in vitro and proliferated on NCGR-COR medium with 1.5 $mg \cdot l^{-1}$ N^6-benzyladenine (BA) and 0.01 $mg \cdot l^{-1}$ indole-3-butyric acid (IBA) (shoot multiplication medium) at 25°C and 16-h photoperiod (25 $\mu mol \cdot m^{-2} \cdot s^{-1}$) as described in chapter 3.

Determination of bacterial contaminants. To detect bacterial contamination in the cultures, shoot tips were streaked on 523 agar plates (Viss et al., 1991). Visible bacterial colonies could be detected as early as 24 hours after inoculation, but plates were held for seven or more days to ensure detection of slow-growing bacteria. The 523 agar plates were used to detect bacterial contamination of cultures before and after antibiotic treatment.

Isolation of bacterial contaminants. A loopful of the contaminant from a 523 plate was mixed into nutrient broth containing 0.8% Difco dehydrated nutrient broth (Difco, Detroit, MI), 1% glucose, and 0.5% yeast extract, then streaked on nutrient agar plates (nutrient broth plus 1.2% Difco Bitek agar) and incubated for three days (seven days for two slow-growing contaminants), at 25°C in the dark. Single colonies were selected and propagated to secure pure cultures. Gram-stain, oxidase,

oxidative/fermentive (O/F), starch hydrolysis (SH), motility, and gelatinase test (Sands, 1990) and colony description were performed on isolates. Three-day old bacterial cultures (seven-day for two slow-growing ones) were used for these tests.

Antibiotic solution preparation. Streptomycin sulfate, gentamicin, and rifampicin (Sigma, St. Louis, MO) were prepared as 10x or 20x concentrated (w/v or v/v) stock solutions in sterile deionized water (rifampicin was dissolved in a small amount of 95% alcohol first) and filter-sterilized using 0.22 μ, 25 mm diameter syringe filters. Stock solutions were diluted in autoclaved half strength liquid MS medium (Murashige and Skoog, 1962) with 0.25 $mg \cdot l^{-1}$ BA at pH 6.9 (LM) as the final antibiotic treatment solutions (Reed et al., in press).

Screening of antibiotics for antibacterial activity. Antibacterial activity of antibiotics was tested on bacteria from 'Nonpareil' (*Corylus* bacterium CB37), 'Tonda Gentile delle Langhe' (CB114), 'Tonda Gentile Romana' (CB475), 'Tonda Gentile delle Langhe' X 'Cosford sel. 3L' (CB494), and 'Willamette' (CB500). A loopful of contaminant isolated from each of the five genotypes was individually inoculated into LM containing an antibiotic: 1000, 500, 250, 100, or 50 $\mu g \cdot ml^{-1}$ streptomycin sulfate or 30, 20, 15, 10, or 5 $\mu g \cdot ml^{-1}$ gentamicin or rifampicin. Bacterial cultures in antibiotic solutions were kept in the dark at 25°C for one week then checked for turbidity caused by bacterial growth. A drop of each bacterial culture was inoculated onto sectors of nutrient agar plates and mixed into liquid nutrient medium then held in the dark at 25°C for four to seven days to check for the survival of bacteria.

Antibiotic treatment of plant materials. In the first experiment 1000 or 1500 $\mu g \cdot ml^{-1}$ streptomycin solution was used to treat infected cultured shoot tips (1-cm

long) and nodal segments (0.5-cm long) of 'Nonpareil', 'Tonda Gentile Romana', and 'Willamette' for six, nine, and 12 days. Three shoot tips and nodal segments of each genotype and each treatment were immersed in individual tubes containing antibiotic solution. In the second experiment, contaminated shoot tips (1-cm long) of hazelnut cvs. Nonpareil, Tonda Gentile Romana, and Willamette were treated with an antibiotic solution for six days. Antibiotic solutions chosen, based on the results of the antibacterial activity tests, were: 15 or 30 $\mu g \cdot ml^{-1}$ gentamicin or rifampicin; 500 $\mu g \cdot ml^{-1}$ streptomycin combined with 15 $\mu g \cdot ml^{-1}$ gentamicin or rifampicin. Five shoot tips of each genotype and each treatment were immersed in individual tubes containing antibiotic solution. Controls (plant tissues grown in LM without antibiotics) were also included in each experiment. Experiments were performed twice.

After antibiotic treatment (six to 12 days), plant tissues were removed from antibiotic solutions, drained on sterile paper towels, the base end of shoots trimmed, and transferred to shoot multiplication medium. At the next transfer, treated shoots were streaked on 523 agar plates to check for residual contamination. Shoots were subcultured without further exposure to the antibiotics and the 523 agar plate was used to check contamination at every transfer. Phytotoxicity of antibiotics was determined visually by checking for necrosis, browning, chlorosis, and morphological changes. The bactericidal effects of antibiotic treatment were evaluated by observing for bacterial growth on 523 agar plates and cloudiness around the shoot base in the multiplication medium. All shoot cultures were kept at 25°C and 16-h photoperiod (25 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

Results and Discussion

Nature of bacterial contaminants

Eight different bacterial contaminants were isolated from the eight hazelnut genotypes. Only one kind of bacteria was isolated from each genotype as indicated by colony morphology on nutrient agar plates and/or reaction to Gram-stain (Table 4.1). We did not determine if there was correlation between bacteria type and plant genotype, but it is likely that bacterial infection was random. Six of the bacteria were Gram-negative and two were Gram-positive. Colonies were visible on nutrient agar plates in three days except CB500 and CB563 which were slow-growing and required about seven days for colonies to be visible. Colony pigmentation varied from light yellow to yellow and pink to pink-red. The results of oxidase, starch hydrolysis (SH), O/F, motility, and gelatinase tests also varied with the isolates (Table 4.1).

CB37 and CB114 had beige pigmentation associated with highly mucoid, moist colonies, characteristics of *Agrobacterium*-related species. Both isolates were positive for oxidase and motility, but starch hydrolysis negative. Colony morphology and biochemical tests matched the characteristics of four isolates from infected mint cultures identified as *Agrobacterium radiobacter* by fatty acid analysis (Buckley et al., in press). CB553 matched the characteristics of *Pseudomonas fluorescens* showing characteristic fluorescence and positive responses to oxidase, motility, and gelatinase tests (Buckley et al., in press). CB565 with yellow colony pigmentation and positive oxidase and motility was characteristic of members of the genus *Xanthomonas*

(Palleroni, 1984). The other isolates were varied in their characteristics and could not be further categorized.

Effect of antibiotics on bacterial isolates

Streptomycin effectively controlled CB475 and CB500 at concentrations higher than 250 $\mu g \cdot ml^{-1}$, but was ineffective on other bacteria even at 1000 $\mu g \cdot ml^{-1}$. Gentamicin was the most effective antibiotic tested, controlling three bacteria (CB37, CB114, and CB494) at as low as 10 $\mu g \cdot ml^{-1}$ and one (CB500) at 30 $\mu g \cdot ml^{-1}$. Only CB475 did not respond to gentamicin at the levels tested. Rifampicin was effective only on CB475 at 30 $\mu g \cdot ml^{-1}$. Our results with gentamicin and rifampicin differed from those of Young et al. (1984) and Cornu and Michel (1987). Young et al. (1984) found that rifampicin was effective for six isolates but gentamicin was effective only for one isolate of the seven bacteria isolated from apple, rhododendron, and Douglas-fir. Cornu and Michel (1987) also reported that rifampicin was effective but gentamicin less so against bacteria isolated from shoot cultures of *Prunus avium*. Phillips et al. (1981) found rifampicin at 50 $\mu g \cdot l^{-1}$ was highly effective in suppressing bacterial growth without phytotoxicity to cell growth of artichoke cultures. The difference in the effect of gentamicin may be due to pH effect and activity of antibiotics at different pH (Buckley et al., in press). Although no single antibiotic was effective for all five bacterial isolates of hazelnut shoot cultures, at least one of the three antibiotics tested was bactericidal for individual isolates. Young et al. (1984) also reported that among rifampicin, tetracycline, cefotaxime, and polymyxin

B no single antibiotic was bactericidal against all of the bacterial isolates from shoot cultures of several woody plants but all the isolates were killed by at least one of the antibiotics.

Effect of antibiotics on controlling bacterial contamination of plant tissues

A combination of streptomycin and gentamicin was effective in eliminating bacteria from 'Nonpareil' (CB37), 'Tonda Gentile Romana' (CB475), and 'Willamette' (CB500) with 50%, 90%, and 40% bacteria free of treated shoots respectively. Treatments with rifampicin or gentamicin singly, and a combination of streptomycin and rifampicin failed to eliminate the bacteria from hazelnut cultures. Contamination was evident in treated cultures 2-3 weeks after transfer from the ineffective antibiotic treatment solutions to shoot multiplication medium. Immersion of infected shoot tips and nodal segments of three cultivars in LM containing streptomycin at 1000 $\mu g \cdot ml^{-1}$ or 1500 $\mu g \cdot ml^{-1}$ for six, nine, or 12 days suppressed the growth of bacteria for a short time but they were again evident after two subcultures (data not shown). Combinations of antibiotics have been used against bacteria from plant tissue culture (Leifert et al., 1991; Young et al., 1984). Young et al. (1984) used a combination of cefotaxime, tetracycline, rifampicin, and polymyxin B at 25, 25, 6, and 6 $\mu g \cdot ml^{-1}$, respectively, to successfully eliminate bacteria from tissue cultures of apple, rhododendron, and Douglas-fir. Leifert et al. (1991) reported that a range of different bacteria were eliminated from contaminated plant tissues of

Hemerocallis, *Choisya*, and *Delphinium* using combinations of gentamicin or streptomycin, carbenicillin and/or cephalothin, and rifampicin.

Phytotoxicity varied among antibiotics and genotypes. 'Nonpareil' tissues were more tolerant of streptomycin than the other genotypes. Treated shoot tips of 'Nonpareil' showed some leaf yellowing but were otherwise healthy and during subsequent growth on antibiotic-free medium the leaves turned green and all shoots grew (Table 4.2); while the other genotypes, especially 'Willamette', showed inhibition of growth as well as leaf yellowing. Inhibition of growth was more severe for treated nodal segments than for shoot tips. Gentamicin at 15 and 30 $\mu g \cdot ml^{-1}$ and the combination of streptomycin and gentamicin were toxic to plant tissues. Leaves became yellow, remained small, and did not grow well but shoot growth resumed after several weeks. Treated shoot tips became brown and soft after six days of treatment with 30 $\mu g \cdot l^{-1}$ rifampicin and some died but others grew well after transfer to shoot multiplication medium. 'Willamette' was more damaged by rifampicin than were 'Nonpareil' and 'Tonda Gentile Romana'.

Both gentamicin and streptomycin belong to the group of aminoglycoside antibiotics. They bind to 30S ribosomal subunits in bacterial cells and inhibit protein synthesis and also might inhibit protein synthesis in chloroplasts and mitochondria in plant tissues (Young et al., 1984), therefore resulting in small and yellow leaves. Phytotoxicity was exhibited with gentamicin (Dodds and Roberts, 1981) and streptomycin at 50 $\mu g \cdot ml^{-1}$ to cell growth of *Helianthus tuberosus* (Phillips et al., 1981) and at 100 $\mu g \cdot ml^{-1}$ to shoot cultures of *Clematis*, *Delphinium*, *Hosta*, *Iris*, and *Photinia* (Leifert et al., 1992). Gentamicin 50 $\mu g \cdot ml^{-1}$ was added in pear culture

medium without harm to the plants (Chevreau et al., 1989). Streptomycin was effective in eliminating bacteria from infected mint cultures with little phytotoxicity (Reed et al., in press). Rifampicin prevents mRNA synthesis by inhibiting DNA-directed bacterial RNA polymerase (Young et al., 1984). Phytotoxicity of rifampicin to mint shoot cultures (Reed et al., in press) and to some clones of cherry (Cornu and Michel, 1987) was reported, but Phillips et al. (1981) and Leifert et al. (1992) reported that rifampicin (50 $\mu g \cdot ml^{-1}$) showed little toxicity for tissues of *Clematis, Delphinium, Hosta, Iris, and Photinia.* These differences may be due partly to treatment methods and likely to genotype variation in susceptibility to antibiotics. Variation among *Corylus* genotypes in susceptibility to antibiotic phytotoxicity was evident in our experiments. 'Willamette' was the most affected and 'Nonpareil' the least for all tested antibiotics.

A proportion of the bacterial contamination encountered in hazelnut shoot cultures (40-90%) was eliminated by combinations of antibiotics used in our study. Other combinations of antibiotics or more than one treatment with antibiotics may also be useful for bactericidal treatments (Reed et al., in press). The bactericidal effect of antibiotics on bacterial isolates was different from that on bacteria established within plant tissues. Gentamicin effectively killed bacterial isolates of 'Nonpareil' (CB37), but it was not effective on bacteria in 'Nonpareil' shoot cultures. Similar results were obtained for streptomycin with bacteria infecting 'Tonda Gentile Romana' (CB475) and 'Willamette' (CB500). Leifert et al. (1991) reported similar results for the bacterium *Pseudomonas maltophilia* in *Delphinium* plant tissues. It may be hypothesized that these differences between the effect of an antibiotic on

isolated bacteria and bacteria harbored within plant tissues are due to 1) insufficient uptake of antibiotic by the plant; 2) bacterial survival in or on plant tissues which the antibiotic did not reach; 3) excessive or unacceptable phytotoxicity at levels effective for bactericidal action; and/or 4) breakdown of the antibiotic by the plant before it is effective against the bacteria.

Table 4.1. Characteristics of bacteria isolated from micropropagated hazelnut shoots.

	CB475[z]	CB494	CB500	CB37	CB114	CB553	CB563	CB565
Gram-stain	+	+	-	-	-	-	-	-
Cell type	Large rod	rod	large rod	rod	small rod	rod	large rod	rod
Colony-color[y]	P	LY	LY	creamy	creamy	Y	PR	Y
shape	round	irregular	round	round	round	irregular	round	irregular
size[x]	small	large	medium	large	large	medium	small	medium
consistency	dry	mucoid	mucoid	mucoid	mucoid	mucoid	mucoid	mucoid
motility	-	-	+	+	+	+	-	+
Oxidase	+	-	+	+	+	+	+	+
O/F[w]	-/-	-/-	-/-	+/-	+/-	+/-	-/-	+/-
SH[v]	-	-	-	-	-	-	-	-
Gelatinase	-	-	-	-	-	+	-	-

[z] NCGR Corylus # from which the bacteria contaminant was isolated.
[y] P, pink; LY, light yellow; Y, yellow; PR, pink-red.
[x] Small: <0.2 cm in diameter; medium: 0.2-0.3 cm; large: >0.3 cm.
[w] Oxidative/Fermentive test.
[v] Starch hydrolysis.

Table 4.2. Recovery of growth of hazelnut shoot cultures five weeks after treatment with streptomycin for six to 12 days followed by transfer to NCGR-COR medium.[z]

Streptomycin (μg/ml)	(day)	Explant	Percent shoot growth[y]		
			Nonpareil (COR37)	T.G.R.[x] (COR475)	Willamette (COR500)
1500	6	Shoot tip	100	100	100
	9	Shoot tip	100	100	0
	12	Shoot tip	100	100	33
	6	Internode	100	67	0
	9	Internode	100	67	0
	12	Internode	67	67	0
1000	6	Shoot tip	100	100	100
	9	Shoot tip	100	100	33
	12	Shoot tip	100	100	67
	6	Internode	100	33	33
	9	Internode	100	67	0
	12	Internode	100	100	0

[z] All controls were contaminated but grew vigorously. All treated cultures failed to show contamination at this transfer, but they were contaminated at the next transfer.
[y] Percentage of treated explants with new shoot growth.
[x] Tonda Gentile Romana.

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Chapter 5

PLANT REGENERATION FROM CULTURED SOMATIC TISSUES OF HAZELNUT

Abstract

Adventitious shoots were regenerated from stem segments or leaf discs of hazelnut in vitro shoot cultures. Five to 10% of stem segments of 'Nonpareil' produced shoots after two to three months of culture on modified MS medium and NCGR-COR medium supplemented with 200 $mg \cdot l^{-1}$ glutamine and combinations of 1 or 5 μM thidiazuron (TDZ) and 0.1 μM α-naphthaleneacetic acid (NAA). Callus derived from stem segments of 'Nonpareil', 'Tonda Gentile Romana', and 'Willamette' and leaf discs of 'Dundee' cultured on medium with TDZ and NAA also produced shoots (buds) after transfer to NCGR-COR medium or modified MS medium. Callus was produced on leaf discs and stem segments of all genotypes tested on medium with TDZ and NAA or N^6-benzyladenine (BA) and 2,4-dichlorophenoxyacetic acid (2,4-D). Explants cultured on medium with BA, zeatin, N^6-(2-isopentenyl)adenine (2iP), and kinetin did not produce either callus or adventitious shoots. Adventitious roots were produced from leaf discs and stem segments on medium with NAA alone or high concentrations of indole-3-butyric acid (IBA) or NAA combined with low concentrations of BA. Somatic embryos were observed on stem segments of 'Tonda Gentile Romana'. Regenerated shoots of

'Nonpareil' and 'Willamette' were multiplied, rooted, and acclimatized in the greenhouse.

Introduction

Adventitious shoot regeneration is a key process for genetic manipulations. For woody species, regeneration from tissues not of seedling origin is often difficult. Plants have been regenerated from leaf discs of micropropagated shoots derived from mature trees of apple (James et al., 1988; Welander, 1988b), pear (Chevreau et al., 1989), cherry (Druart, 1990; Antonelli and Druart, 1990), and quince (Dolcet-Sanjuan et al., 1991). Somatic embryogenesis from hazelnut (*Corylus avellana*) cotyledonary node tissue was reported (Perez et al., 1983; Perez et al., 1986), but neither somatic embryogenesis or adventitious shoot regeneration have been achieved with tissues not of embryonic or seedling origin. In this study, stem segments, leaves, and petioles from micropropagated shoots originating from mature hazelnut trees were used to study the influence of culture medium, explant type, and growth regulators on regenerative capacity.

Materials and Methods

Shoot cultures of *Corylus avellana* cvs. Tonda Gentile Romana, Willamette, Nonpareil, and rootstock Dundee were established from mature trees and maintained on NCGR-COR medium with 6.6 μM N^6-benzyladenine (BA) and 0.05 μM indole-3-butyric acid (IBA) (Yu and Reed, 1993; chapter 3). Leaves, petioles, and internodal stem segments from shoot tips of three- to four-week old in-vitro shoots were used for callus production and shoot regeneration. Young leaves were dissected with transverse incisions leaving the sections together at one margin (Dolcet-Sanjuan et al., 1991). Five leaves were placed in each petri dish with the abaxial side on the medium. Stem segments (0.3-0.5 cm in length) were prepared so that all axillary buds were excluded. Stem segments and petioles were placed vertically on the medium with the basal end down; six to eight explants were placed in each petri dish.

The culture media for regeneration studies were NCGR-COR medium (chapter 3) and modified MS medium (Murashige and Skoog, 1962) with one-half the concentration of MS mineral salts and with 30 $g \cdot l^{-1}$ glucose as the carbon source. Glutamine (200 mg/l) was added to some media as described below. The pH was adjusted to 5.2 prior to the addition of 5 $g \cdot l^{-1}$ Difco Bitek agar (Detroit, MI) and autoclaving. Medium was autoclaved at 121°C and dispensed into petri dishes (20 ml/dish). Cultures were kept at 25°C in the dark for the first three weeks and with a 16-h photoperiod (25 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for the remainder of the culture period.

Combinations of cytokinins [thidiazuron (TDZ), BA, zeatin, N^6-(2-isopentenyl)adenine (2iP), and kinetin] with α-naphthaleneacetic acid (NAA); BA

with IBA or 2,4-dichlorophenoxyacetic acid (2,4-D) were tested as described below. TDZ was dissolved in dimethylsulfoxide (DMSO) and added to the medium after autoclaving (at 0.1% v/v) (Schmitz and Skoog, 1970). Other growth regulators were added before autoclaving.

Experiment 1. Leaf discs, stem segments, and petioles (15 each) of cvs. Nonpareil and Tonda Gentile Romana were cultured on modified MS medium with 1 to 50 μM BA, 2iP, kinetin, zeatin, or TDZ combined with 1 μM NAA.

Experiment 2. TDZ concentrations of 0.33, 1, 3.3, 10, or 33 μM combined with 0, 1, 3.3, or 10 μM NAA in modified MS medium and NCGR-COR medium were used. Ten leaf discs and 20 stem segments of 'Nonpareil' and 'Tonda Gentile Romana' were used per treatment and the experiment was repeated once.

Experiment 3. The effects of modified MS medium and NCGR-COR medium supplemented with 200 $mg \cdot l^{-1}$ glutamine, TDZ 1, 5, 25, or 50 μM, and 0.1 μM NAA, were determined using stem segments and leaf discs of 'Nonpareil', 'Tonda Gentile Romana', 'Willamette', and 'Dundee', with 20 stems and 10 leaf discs of each genotype per treatment. Two replicate experiments were performed.

To determine the ability of callus to proliferate or form shoots, callus derived from stem segments and leaf discs on modified MS medium was transferred to NCGR-COR medium containing 6.6 μM BA and 0.05 μM IBA or modified MS medium supplemented with 22 μM BA and 0.5 μM NAA or IBA, or 0.5 μM BA and 5.3 or 10.6 μM NAA.

Experiment 4. Leaf discs and stem segments of 'Nonpareil', 'Tonda Gentile Romana', 'Willamette', and 'Dundee' were cultured on modified MS medium with

200 $mg \cdot l^{-1}$ glutamine and combinations of 2,4-D (4.5, 9 μM), IBA (4.9, 9.8 μM), or NAA (5.3, 10.6 μM) with BA (0.5 μM) for the first four weeks and then transferred to the same basal medium with 6.6 μM BA and 0.6 μM IBA or with no growth regulators. Ten explants of each genotype were used per treatment.

Explants with adventitious roots were transferred to NCGR-COR medium containing 6.6 μM BA and 0.05 μM IBA to determine whether shoots could be obtained.

Shoot multiplication and plant establishment. Regenerated shoots were multiplied on NCGR-COR medium containing 6.6 μM BA and 0.05 μM IBA. Shoots were rooted ex vitro by dipping the basal end of shoots into a 1 mM IBA solution for one min and planting them in a mix of vermiculite and perlite (1:1). They were placed in a mist bed for two weeks and transferred to the greenhouse bench for acclimatization.

Results and Discussion

Adventitious shoot formation

Medium with glutamine, TDZ, and NAA induced callus formation and shoot regeneration from leaf discs and stem segments. In contrast, no callus or adventitious shoots were produced from petioles or any explants cultured on medium with BA, 2iP, zeatin, or kinetin at any concentration combined with 1 μM NAA. All explants on medium with kinetin turned brown and died.

On medium with glutamine (200 $mg \cdot l^{-1}$), TDZ (1, 5, 25, and 50 μM), and NAA (0.1 μM), leaf discs expanded three to five times in size and stem segments became swollen. Callus was produced from the cut surfaces after two to three weeks in the dark and continued to increase when cultures were exposed to the light, finally covering the entire explant. Callus of 'Tonda Gentile Romana', 'Nonpareil', and 'Willamette' was green, compact and nodular with some pink pigmentation; callus from leaf discs of 'Dundee' was green-yellow and friable with primordia-like structures; and callus from stem segments of 'Dundee' was light green and friable but turned brown after eight weeks.

The calli grew very slowly after about six weeks on the same medium. They did not proliferate after transfer to medium with high NAA (5.3 or 10.6 μM) or IBA (4.9 or 9.8 μM) and low BA (0.5 μM). Adventitious shoots were produced on 5-10% of stem segments of 'Nonpareil' after two to three months of culture on modified MS

medium or NCGR-COR medium supplemented with 200 $mg \cdot l^{-1}$ glutamine and combinations of 1 or 5 μM TDZ and 0.1 μM NAA (Table 5.1).

Callus of 'Nonpareil', 'Tonda Gentile Romana', and 'Willamette' remained green after transfer to NCGR-COR medium with 6.6 μM BA and 0.05 μM IBA or modified MS medium with either NAA or IBA at 0.5 μM and BA at 22 μM or without growth regulators. Some of these calli produced shoots two weeks after transfer. These calli were derived originally from stem segments of 'Nonpareil', 'Tonda Gentile Romana', and 'Willamette' and leaf discs of 'Dundee' cultured on modified MS medium with 1, 5, or 50 μM TDZ and 0.1 μM NAA. No adventitious shoots were produced from calli derived from leaf discs of 'Nonpareil', 'Tonda Gentile Romana', and 'Willamette' or from stem segments of 'Dundee' (Table 5.1).

Usually only one shoot was produced on a regenerating explant of 'Nonpareil' and 'Willamette', although some of the explants had four to seven buds (Fig. 5.1). Buds produced from 'Tonda Gentile Romana' and 'Dundee' did not elongate and multiply further. It was not determined whether adventitious buds were produced from the surface of the callus or the original explants.

The combination of BA and NAA was reported as very effective in leaf disc regeneration of apple (James et al., 1988; Welander, 1988). For *Betula*, 2iP and zeatin were effective (Simola, 1985). In our experiments, shoot regeneration occurred only from explants originally cultured on medium with TDZ. TDZ was very effective for leaf disc regeneration of *Cydonia oblonga* (Dolcet-Sanjuan et al., 1991) and pear (Chevreau et al., 1989).

Somatic embryos (globular, torpedo shape) were observed in one replication in callus produced on stem segments of 'Tonda Gentile Romana' cultured on modified MS medium with 200 $mg \cdot l^{-1}$ glutamine, 25 or 50 μM TDZ and 0.1 μM NAA. These somatic embryos were transferred to NCGR-COR medium with 6.6 μM BA and 0.05 μM IBA or without growth regulators, but they did not develop further.

Adventitious roots were produced from leaf discs and stem segments of 'Nonpareil' and 'Tonda Gentile Romana' cultured on medium with 0, 1, 3.3, or 10 μM NAA. Adventitious roots were also produced from leaf discs and stem segments of 'Nonpareil', 'Tonda Gentile Romana', 'Willamette', and 'Dundee' cultured on medium with IBA (4.9, 9.8 μM) or NAA (5.3, 10.6 μM) and 0.5 μM BA. Two to three roots were produced from different locations on the same explant. These roots were produced from the stem, central vein of leaves and leaf petioles connected to leaves without callus production. The rooted tissues did not produce shoots after they were transferred to medium with 6.6 μM BA and 0.6 μM IBA and they eventually died.

Perez et al., (1983 and 1986) obtained somatic embryogenesis when cotyledonary node segments of hazelnut were cultured for 20 days on medium with 5 μM IBA and 0.5 μM BA followed by 20 days on medium with 0.5 μM IBA and 5 μM BA. They found that BA induced plant regeneration via asexual embryogenesis on cotyledonary nodes. With similar growth regulator combinations, we observed that leaf discs and stem segments produced little callus on cut surfaces and adventitious roots developed from primary explants on medium with low BA concentrations and high concentrations of IBA or NAA, and callus stopped growing

and turned brown when transferred to medium with 6.6 μM BA and 0.6 μM IBA. The difference between our results and those of Perez et al. may be due to the differences in maturity of materials and the basal media used.

On medium with 2,4-D and BA, leaf discs expanded slightly and whole leaf discs and stem segments were callused with soft, light yellow callus. A pink pigment was produced on the surface of the callus.

Several factors can influence regeneration frequency, including hormonal balance, mineral nutrients, light conditions, carbon source and concentration, and physiological condition of leaves and stem segments (Welander, 1988; James et al., 1988; Druart, 1990; Antonelli and Druart, 1990; Dolcet-Sanjuan et al., 1991). Trials on these factors may increase the hazelnut regeneration frequency.

Shoot multiplication and plant establishment

Shoots regenerated from stem segments of 'Nonpareil' and 'Willamette' were proliferated, rooted, and acclimatized with high rooting frequency (100% for 'Willamette' and 80% for 'Nonpareil') and survival rate (100% for 'Willamette' and 90% for 'Nonpareil').

Conclusions

Five to 10% of stem segments of 'Nonpareil' regenerated adventitious shoots on medium with 200 $mg \cdot l^{-1}$ glutamine, 1 or 5 μM TDZ, and 0.1 μM NAA. Adventitious shoots (buds) were also produced from stem segments of 'Nonpareil', 'Tonda Gentile Romana', and 'Willamette' and leaf discs of 'Dundee' cultured on modified MS medium with glutamine, TDZ, and NAA followed by culture on NCGR-COR medium with BA and IBA, or modified MS medium with BA and IBA, or NAA or without growth regulators. Adventitious roots were produced on medium with NAA alone or with high levels of IBA or NAA combined with BA (0.5 μM). This provides a starting point for improving the plant regeneration frequency to a level useful for genetic manipulation.

Table 5.1. Adventitious shoot production from stem segments and leaf discs of four hazelnut cvs., Nonpareil, Tonda Gentile Romana (T.G.R.), Willamette, and Dundee on NCGR-COR or modified MS medium with 200 $mg \cdot l^{-1}$ glutamine, TDZ, and 0.1 μM NAA.[z]

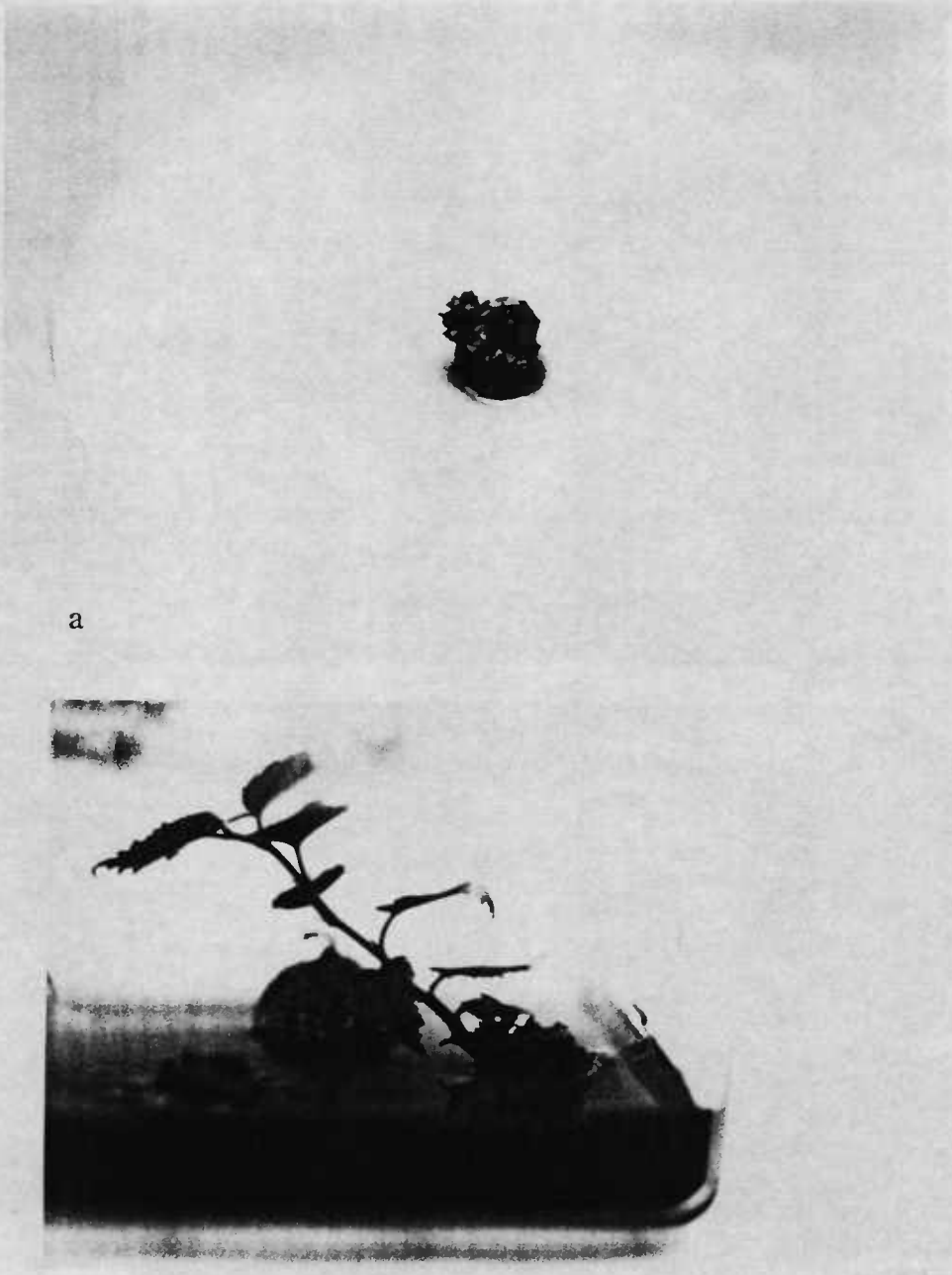
Genotype	TDZ (μM)	Shoots on modified MS		Shoots on NCGR-COR	
		Stem	Leaf disc	Stem	Leaf disc
Nonpareil	1	3[y]	0	2[w]	0
	5	4[y]	0	0	0
	25	0	0	0	0
	50	0	0	0	0
T.G.R.	1	0	0	0	0
	5	2[y]	0	0	0
	25	0	0	0	0
	50	0	0	0	0
Willamette	1	0	0	0	0
	5	2[x]	0	0	0
	25	0	0	0	0
	50	1[x]	0	0	0
Dundee	1	0	0	0	0
	5	0	2[x]	0	0
	25	0	0	0	0
	50	0	2[x]	0	0

[z] Number of stem segments (n = 40) or leaf discs (n = 20) which produced adventitious shoots.

[y] Adventitious shoots produced both on the medium indicated and after transfer to either NCGR-COR medium with 6.6 μM BA and 0.05 μM IBA or modified MS medium with 22 μM BA and 0.5 μM IBA.

[x] Adventitious shoots produced after transfer to either NCGR-COR medium with 6.6 μM BA and 0.05 μM IBA or modified MS medium with 22 μM BA and 0.5 μM IBA.

[w] Adventitious shoots produced on original medium.



a

b

Fig. 5.1. Adventitious buds (shoots) regenerated from 'Nonpareil' stem segment cultured on modified MS medium with 200 $mg \cdot l^{-1}$ glutamine, 5 μM TDZ, and 0.1 μM NAA. a) buds; b) elongated shoots.

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APPENDIX

Appendix

MEIOSIS IN *CORYLUS* INTERSPECIFIC HYBRIDS

Abstract

Several interspecific hybrids of hazelnut (*Corylus*) were produced in the breeding program at Oregon State University. Their usefulness for further breeding is limited because they are characterized by a high percentage of blank nuts. In this study, meiotic behavior in pollen-mother cells of the interspecific hybrids, *C. cornuta* var. *californica* #3 X *C. avellana* 55-129, *C. cornuta* var. *californica* #8 X *C. avellana* 55-129, and *C. heterophylla* HF13 X *C. avellana* 55-129, was investigated. Meiotic abnormalities were observed in all the hybrids studied (varying from 26 to 48% of the cells). Varying numbers of univalents, trivalents, and quadravalents were present at the first metaphase. Early disjunction of bivalents, chromosome lagging, and unequal chromosome distribution were observed. Univalents were also observed in the pollen mother cells (PMCs) of some of the parents: *C. cornuta* var. *californica* #3, *C. cornuta* var. *californica* #8, and *C. heterophylla* HF13. Low frequency of quadrivalents was observed in PMCs of *C. avellana* 55-129. The production of blank nuts was correlated with the meiotic irregularities.

Introduction

Corylus avellana L. is the main species used for commercial hazelnut production. Hybridization of *C. avellana* with other species is necessary when the desired trait such as cold-hardiness, resistance to eastern filbert blight, or non-suckering growth habit cannot be found in *C. avellana* (Thompson et al., in press). Several interspecific hybrids have been produced in the hazelnut breeding program at Oregon State University. Blank nuts (i.e. nuts devoid of normal kernels) prevail in some interspecific hybrids and are also present in some cultivars (Thompson et al., in press). Blank nuts may result from defective embryo sacs, inviable eggs, failure of fertilization, or embryo abortion at varying stages of development. McKay (1966) first suggested that high male sterility due to meiotic irregularities in microsporogenesis might be associated with high female sterility (i.e. blank nuts). However except for one male-sterile plant with 85% blank nuts, his own evidence did not support this hypothesis. Salesses and Bonnet (1988) reported the association of heterozygous translocations with reduced male fertility but did not mention female sterility. The high frequency of blank nuts in 'Barcelona', one of the cultivars having both reduced pollen fertility and a translocation heterozygote, might be caused by cytological abnormalities occurring in megasporogenesis (Thompson et al., in press).

In this study, meiotic behavior in pollen-mother cells of *Corylus* hybrids was investigated to determine if cytological abnormalities were associated with the high frequency of blank nuts found in these hybrids.

Materials and Methods

Plants used in this study were hybrids of *C. cornuta* var. *californica* #3 X *C. avellana* 55-129 (Hybrid of Tonda Gentile delle Langhe X Extra Ghiaghli) (hybrids No. 77, 80, and 82), *C. cornuta* var. *californica* #8 X *C. avellana* 55-129 (83, 90, and 92), *C. heterophylla* HF-13 X *C. avellana* 55-129 (95, 97, and 99), and the four parents.

Hazelnut catkins (male flowers) were collected from early to late September in 1991 and from early to late August in 1993, when they were about 1.5 cm in length and 0.5 cm in diameter and the anthers were still green but beginning to turn yellow. The developmental stage of anthers varied among scales within a catkin. Catkins were fixed in modified Carnoy's solution (6 chloroform : 3 95% ethanol : 1 glacial acetic acid) for 24 to 48 h and rinsed with 70% ethanol twice, each for 1 h, then stored in 70% ethanol at 4°C. Slides were prepared by placing anthers on a slide, adding a drop of Snow's alcoholic hydrochloric acid-carmine stain (Snow, 1963) and squashing. Before the cover slip was put on, the anther wall debris was removed and a small drop of 45% acetic acid was applied. The slide was placed on a hot metal plate (60°C) for about 1 sec. and then pressure applied. Chromosome configurations were examined in pollen mother cells (PMCs) at diakinesis, metaphase I, and/or anaphase I under a light microscope.

Results and Discussion

The average chromosome pairing configurations in PMCs of hybrids and parents are summarized in Table A.1 and the percentage of PMCs exhibiting various pairing configurations is given in Table A.2. The chromosome number of these hybrids and their parents was 2n = 2x = 22. Meiotic behavior of hybrids differed from that of the parents. Univalents, trivalents, and quadrivalents as well as bivalents were observed in the hybrids whereas the parents showed only bivalents and univalents or quadrivalents.

The mean number of univalents per cell was the highest in the hybrids of *C. cornuta* #8 X *C. avellana* (1.48 univalents) and the lowest in the hybrids of *C. heterophylla* X *C. avellana* (0.64 univalents). The mean number of bivalents per cell was the highest in *C. heterophylla* X *C. avellana* hybrids (10.40 bivalents) and the lowest in *C. cornuta* #8 X *C. avellana* hybrids (9.91 bivalents). The mean number of multivalents per cell was the highest in *C. cornuta* #3 X *C. avellana* hybrids (0.16 trivalents and 0.12 quadrivalents) and the lowest in *C. heterophylla* X *C. avellana* hybrids (0.03 trivalents and 0.12 quadrivalents).

All hybrids had lower percentages of PMCs with 11 bivalents than their parents. *C. heterophylla* and *C. avellana* hybrids appeared to have the highest mean percentage of PMCs with 11 bivalents and the lowest mean percentage of PMCs with some univalents and the lowest percentage of PMCs with multivalents among the three groups of hybrids. *C. cornuta* #3 and *C. avellana* hybrids had the lowest mean percentage of PMCs showing 11 bivalents and highest mean percentage of

multivalents. *C. cornuta* #8 and *C. avellana* hybrids had a lower mean percentage of PMCs showing multivalents than *C. cornuta* #3 and *C. avellana* hybrids and a higher percentage of univalents than *C. heterophylla* and *C. avellana* hybrids, although there was some overlap among the three groups of hybrids.

No parents consistently had 11 bivalents at diakinesis or metaphase I (Table A.1, A.2). *C. avellana* 55-129 had a high percentage of PMCs with 11 bivalents (92%) and the average bivalents per cell was 10.84. Univalents were observed in the PMCs of *C. cornuta* #3 (0.32/cell, 12% PMCs), *C. cornuta* #8 (0.42/cell, 18.6% PMCs), and *C. heterophylla* (1.5/cell, 20% PMCs) but not in those of *C. avellana* 55-129. Quadrivalents were observed in pollen mother cells of *C. avellana* 55-129 (0.08/cell, 8% PMCs), but not in those of *C. cornuta* #3, *C. cornuta* #8, and *C. heterophylla.*

Early disjunction of bivalents at first metaphase and chromosome lagging at anaphase were observed in some PMCs of all hybrids. Unequal chromosome distribution was occasionally observed at second metaphase.

Univalents in PMCs of the F_1 hybrids could be due to early disjunction of bivalents, the unpaired chromosome from a trivalent and a univalent resulting from a heterozygous translocation, a failure of crossing over in the small chromosomes, and/or lack of chromosome homology between the parents. The formation of a quadrivalent or a trivalent plus a univalent at first metaphase may due to a heterozygous translocation. The univalents in the parents may be the result of bivalent early disjunction or may be due to a failure of crossing over in the small chromosomes. The quadrivalents in *C. avellana* 55-129 may be inherited from one

of its parents, Tonda Gentile delle Langhe, which was reported to be a translocation heterozygote (Salesses, 1973). It is presumed that all the hybrids and *C. avellana* 55-129 may be a translocation heterozygote.

Unpaired chromosomes and multivalents often result in unequal chromosome distribution or chromosome loss at anaphase. Therefore unbalanced, inviable gametes result and fertility is reduced. Early disjunction of bivalents should not affect fertility. It is presumed that similar irregularities occur in megasporogenesis and produce defective eggs, and thus blank nuts.

These hybrids produced high percentages of blank nuts (Table A.3). *C. heterophylla* X *C. avellana* 55-129 hybrids had the lowest mean percentage of blank nuts (35%) and *C. cornuta* #3 X *C. avellana* 55-129 hybrids the highest (42%). The mean percentage of blank nuts was directly related to the mean percentage of irregularity of chromosome behavior at meiosis of PMCs. Therefore the high percentage of blank nuts in these hybrids may be the result of irregular chromosome behavior at meiosis.

Table A.1. Chromosome configurations at meiosis in pollen mother cells (PMCs) of hazelnut interspecific hybrids and their parents.

	Total PMCs	Chromosome configuration[z] I	II	III	IV
C. cornuta #3 X *C. avellana*					
No.77	70	0.90(0-6)[y]	10.10(5-11)	0.13(0-2)	0.13(0-2)
No.80	52	0.62(0-4)	10.23(7-11)	0.15(0-2)	0.12(0-1)
No.82	46	1.63(0-6)	9.63(3-11)	0.21(0-3)	0.12(0-1)
Mean	56	1.05	9.99	0.16	0.12
C. cornuta #8 X *C. avellana*					
No.83	50	1.60(0-8)	9.84(7-11)	0	0.20(0-2)
No.90	56	1.04(0-6)	10.14(7-11)	0.11(0-1)	0.07(0-1)
No.92	37	1.81(0-8)	9.76(4-11)	0.08(0-2)	0.11(0-2)
Mean	48	1.48	9.91	0.06	0.12
C. heterophylla X *C. avellana*					
No.95	55	0.31(0-4)	10.60(8-11)	0.02(0-1)	0.11(0-1)
No.97	50	1.38(0-6)	10.14(7-11)	0.06(0-1)	0.04(0-1)
No.99	65	0.22(0-2)	10.46(9-11)	0	0.22(0-1)
Mean	57	0.64	10.4	0.03	0.12
C. avellana	50	0	10.84(9-11)	0	0.08(0-1)
C. cornuta #3	60	0.32(0-4)	10.84(9-11)	0	0
C. cornuta #8	62	0.42(0-6)	10.79(8-11)	0	0
C. heterophylla	40	1.50(0-8)	10.30(6-11)	0	0

[z] I, univalent; II, bivalent; III, trivalent; IV, quadrivalent.
[y] Mean chromosome configuration per cell (range of the chromosome configuration in PMCs).

Table A.2. Percentage of pollen mother cells (PMCs) showing various pairing configurations in hazelnut interspecific hybrids and their parents.[z]

	Total PMCs	Percentage of PMCs with			
		I	11 II	(III + IV)	Irregularity[y]
C. cornuta #3 X *C. avellana*					
No.77	70[x]	26	57	17	43
No.80	52	19	60	21	40
	(111)	(9)	(73)	(18)	(27)
No.82	46	28	52	20	48
	(59)	(20)	(51)	(29)	(49)
Mean	56	24	56	19	44
C. cornuta #8 X *C. avellana*					
No.83	50	20	64	16	36
No.90	56	29	53	18	47
No.92	37	24	65	11	35
	(50)	(26)	(62)	(12)	(38)
Mean	48	24	61	15	39
C. heterophylla X *C. avellana*					
No.95	55	13	74	13	26
	(50)	(0)	(72)	(28)	(28)
No.97	50	28	62	10	38
No.99	65	11	68	21	32
	(76)	(8)	(61)	(31)	(39)
Mean	57	17	68	15	32
C. avellana	50	0	92	8	8
C. cornuta #3	60	12	88	0	12
C. cornuta #8	62	19	81	0	19
C. heterophylla	40	20	80	0	20

[z] I, univalent; II, bivalent; III, trivalent; IV, quadrivalent.
[y] The total percentage of PMCs with irregular chromosome configurations.
[x] Data in parenthesis from 1991 observation, the others from 1993 observation.

Table A.3. Percentage of blank nuts of hazelnut interspecific hybrids and some of the parents.[z]

	Percentage of blank nuts		
	1990	1991	Mean
C. cornuta #3 X *C. avellana*			
No.77	-	36	36
No.80	-	38	38
No.82	44	59	52
Mean			42
C. cornuta #8 X *C. avellana*			
No.83	24	40	32
No.90	-	36	36
No.92	54	46	50
Mean			39
C. heterophylla X *C. avellana*			
No.95	-	25	25
No.97	24	19	22
No.99	49	30	40
Mean			35
C. avellana			15
C. heterophylla			14

[z] Data from Dr. S.A. Mehlenbacher. Data from *C. cornuta* var. *californica* not available (% blank nuts not determined).

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